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ORIGINAL PAPER

Impact of different environmental stress factors upon the expression of adherence ability in *Escherichia coli* strains isolated from marine waters

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Abstract:
Objective. The purpose of the present study was to investigate the expression of cell-associated virulence factorsin 100*E. coli*strains isolated from Black Sea Coast, in different cultivation conditions simulating different environmental stress factors.**Material and methods.**The bacterial adherence to HeLa cells was investigated in variable incubation temperatures, salinities, pH and O_2concentration, followed by PCR detection of genes codifying proteins involved in adherence:*aggR*,*aafI /II*and*EAST /1*.**Results.**The tested strains developed the ability to grow at 22°C, 37°C, 44°C, irrespective to the salinity, pH and glucose concentration, in aerobic and anaerobic conditions.The adherence capacity to a cellular substratum with a predominantly localized pattern was significantly influenced by the variation of the NaCl concentration. An adherence index of 100% was noticed at pH of 7.2 and9.6 at 37°C, in aerobiosis.The expression of various adherencepatternswas highlighted for bacteria grown atacidpH (5.2) and respectively basic (9.6).

Key words*:* decompensate *E. coli*, PCR, cell-associated, environmental stress factors.

Introduction

Fecal contamination of surface waters is a serious environment and public health issue. In complex systems, fecal pollution can come from several sources, including wastewater discharge, agricultural and urban pluvial spills. Identifying and eliminating the source of contamination is not simple, because evaluating fecal pollution generally relies on a limited number of surface water samples to measure the density of fecal indicators (Gordon et al. 2002; Byappanahalli et al., 2003).

Escherichia coli is a fecal coliform bacteria that exists in the animal and human intestine. The presence of the *Escherichia coli* bacteria in water is a strong indicator of recent contamination with animal dejection or wastewaters. During rains, melting snow or other precipitation, these bacteria can be carried in bays, rivers, lakes or groundwater, and when these waters are used as drinking water and not treated or inadequately treated, the bacteria can get into the drinking water (Llopis et al., 2004).

Numerous studies have indicated that *Escherichia coli* can resist in benthos, being subsequently detected in surface waters (Torrella et al., 2003). Residual pollution persists even at low temperatures, where fecal coliform levels in wastewater initially rapidly decrease, but then stabilize at 1% to 10% of the total initial population. Moreover, *Escherichia coli* isolated from septic tanks proved to be less genetically variable, forming a distinct clone, unlike *Escherichia coli* streams isolated from inhabitants of households serviced by these systems (McLellan , 2004).

Although most *Escherichia coli* streams are nonpathogenic and live in healthy human and animal intestines, there still are some *Escherichia coli* streams that may express virulence factors acquired from pathogenic species, factors responsible for the occurrence of severe clinical forms of infection.

Material and methods

100 environmental *Escherichia coli* were isolated in Constantza, Romania fromsea water.The isolation and identification of these strains was based on filter membrane method, according to SR ISO 9308-1 2000. This technique consists in filtering 100 ml water sample using a filter membrane of 47mm diameter. The membrane is applied on Lactose TTC medium poured in 47 mm diameter Petri plates. After 48 hours incubation at 44°C, *Escherichia coli* will develop yellow colonies on the membrane.Oxidase and indole production test were performed additionally for the identification of *Escherichia coli* strains.

The influence of different stress factors on cell-associated virulence factors

Bacterial suspensions adjusted to 0.5 McFarland standard turbidity performed in phosphate buffer saline (PBS) from 24h cultures were used for inoculation of 1 ml broth and then incubated at five different temperatures (4ºC, 22ºC, 37ºC, 44ºC and 56ºC), in aerobic and respectively anaerobic conditions for 24h. The same suspensions were used for inoculation of 1 ml broth with 3 different pH values (5.0, 7.2 and 9.6), 2 different glucose concentrations (1.5% and 3%), and 9 different concentrations of Na Cl (0%, 0.5%, 2%, 3%, 4%, 5%, 6%, 7%, and 10%).

The adherence capacity to the biotic substratum (HeLa cells) was investigated by using Cravioto method. In this purpose 1 ml bacterial suspension prepared from cultures obtained in different experimental conditions was inoculated on (80%) confluent cellular layer of HeLa-2 cells. After an incubation of 2 h at 37°C, the bacterial suspension was discarded and the cell culture washed and colored by Giemsa method. The adhesion was microscopically examined for the identification of the adhesion patterns (i.e. diffuse, localized and aggregative) and for the quantification (+, ++, +++, ++++) points of view (Lazar, 2003).

Molecular investigation

To highlight the genes codifying proteins involved in adherence: *aggR*, transcriptional activator of genes for type I expression fimbrias in enteroaggregative*E. coli* (EaggEC), the genes for afimbrialadhesins (*aafI / II*), and genes involved in the synthesis of some toxins, i.e. thermostable enterotoxin (*EAST / 1*), we applied multiplex – PCR techniques.

For this reaction there were used following pairs table 1:

Table 1 - Sequence pairs of primers used for highlighting genes*aaf*I, *aggR* and*EAST*/1 (Balotescu, 2004)

Gene	Primer sequence	t°alignment	Size amplicons
*aaf*I	Forward - 5'-TGGGATACTCTCAGG-3' Reverse - 5'-TACCAGATATAAATATAGGG-3'	52°C 60 sec	450 pb
aggR	Forward -5'-CGATGTATACACAAAAGAAGGA-3' Reverse - 5'-GCCTAATGAAATATGATGGTACT-3'	56°C 60 sec	640 pb
EAST/1	Forward.-5'CCATCAACACAGTATATCCGA-3' Reverse - 5'-GGTCGCGAGTGACGGCTTTGT-3'	56°C 60 sec	111 pb

Amplification mixture was obtained as illustrated in table 2.

Table 2 - Amplification reaction components.

Reagents	Volume/sample	Final concentration
buffer green 10x	5.0µl	1x
$MgCl_2$ 25mM	3.0µl	2.5mM
mix dNTP 10mM	8.0µl	3.2mM
primer 1– 20µM	3.0µl	0.04µM
primer 2– 20µM	3.0 lµ	0.04µM
TaqPol 5U/µl	0.5µl	0.5U
A.D.D.	17.5µl	-
Cell lysates	10 µl	-
Total volume of reaction	50.0µl	

Reaction conditions for multiplex PCR:

- 1 cycle to 96°C timefor 5 min
- 35 cycle:
  - 96°C timefor 1.0 min
  - 43°C timefor1.0 min
  - 72°C timefor90 sec
- 1 cycle to 72°C timefor10 min.

Results and discussion

Normal biological activity of microorganisms is strongly influenced by physical and chemical environmental conditions. The biological activity is greater when environmental conditions are optimal to the species needs, when metabolic reactions involved in the processes of growth and multiplication take place normally. Optimal conditions for microorganisms living in natural environments are very rare, but bacteria compensate by high resistance to adverse conditions and a high capacity for adaptation, compared with higher organisms (Bryan, 1985; Cardonha et al. 2005).

Bacteria growth in liquid media stimulated their adherence on the cellular substratum, represented by HeLa cells, indicating the fact that bacterial development in the liquid medium could favor the initiation of an infectious process (fig. 1).

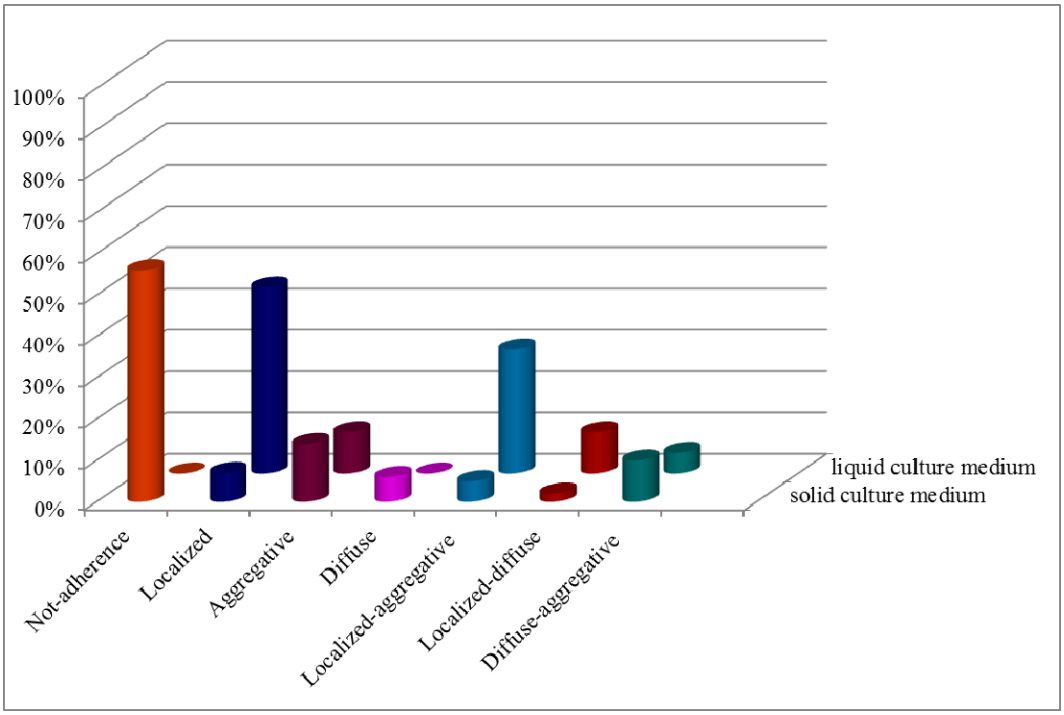

Fig. 1 - The influence of the consistency of the culture medium on the bacterial adherence capacity to the HeLa cellular substratum in *E. coli* strains isolated from sea water.

Temperature is one of the most important environmental factors influencing growth and survival of organisms, the rate of biochemical reactions in cells increasingproportionally, but nucleic acids and other cellular components are sensitive to high temperatures above a certain level and can be irreversibly inactivated. Unlike the extreme temperatures that suspend any antimicrobial

activity, the moderate ones are allowing the normal metabolic processes and growth and multiplication of microorganisms (Barras and Fontecave, 2011).

As you can observe in figure 2, the maximum bacterial adherence capacity to the HeLa cellular substratum was expressed at a temperature of 37°C. Regardless of the incubation temperature, the most frequently expressed adherence pattern was the localized one.

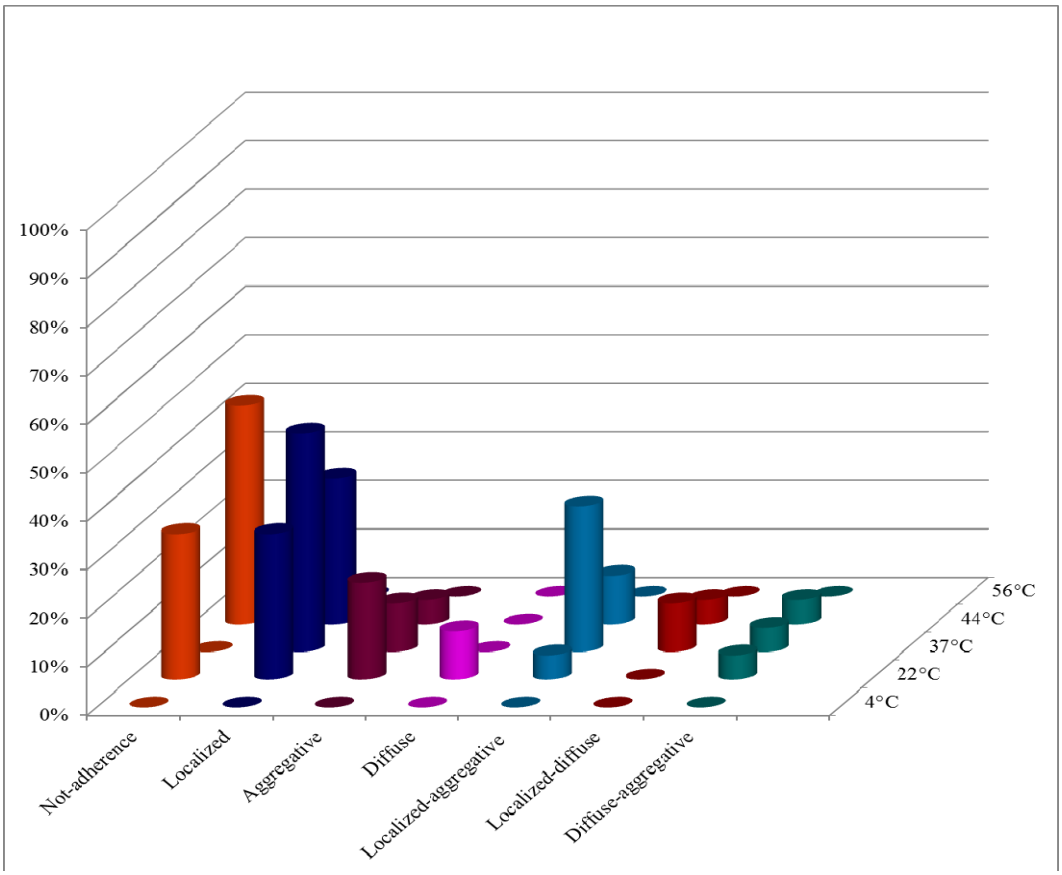

Fig. 2 - The influence of temperature on the bacterial adherence capacity to the HeLa cellular substratum (%), in aerobiosis conditions, in *E. coli* strains isolated from sea water.

As it can be observed in figure 3, bacterial adherence capacity to the HeLa cellular substratum, in anaerobiosis conditions was also best expressed at 37°C. Regardless of the incubation temperature, the most frequently expressed adherence pattern was the localized one.

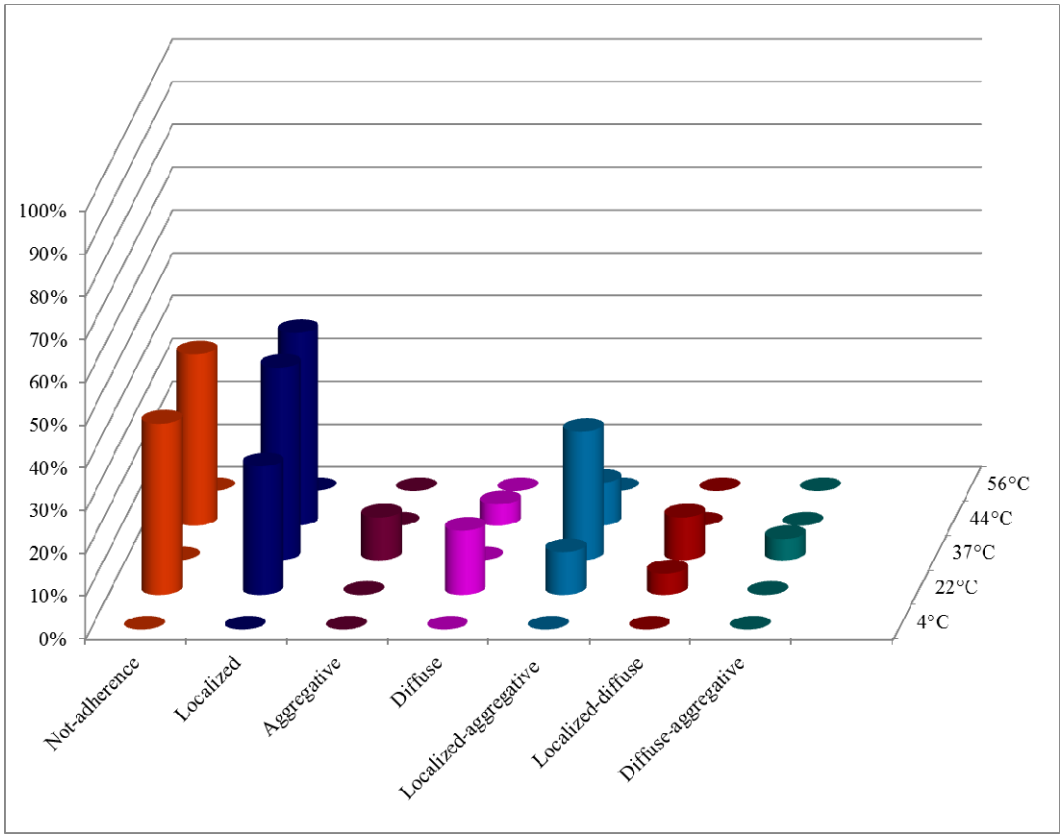

Fig. 3 - The influence of temperature on the bacterial adherence capacity to the HeLa cellular substratum (%), in *E. coli* strains isolated from sea water, inanaerobiosis conditions.

Growing bacteria in aerobiosis and anaerobiosis conditions indicated that the presence/absence of O_2 does not influence the bacterial adherence capacity to the HeLa cellular substratum, when the strains are cultivated 37°C (fig. 4).

It should be noted that at 22°C, the absence of O_2 induced a decrease of the bacterial adherence capacity to the HeLa cellular substratum, and the aggregative pattern was not expressed (fig. 4).

Bacterialgrowth at 44°C in anaerobiosis conditions induced a slight decrease of the bacterial adherence capacity to the HeLa cellular substratum, revealing the following adherence patterns: localized, diffuse and localized-aggregative (fig. 4).

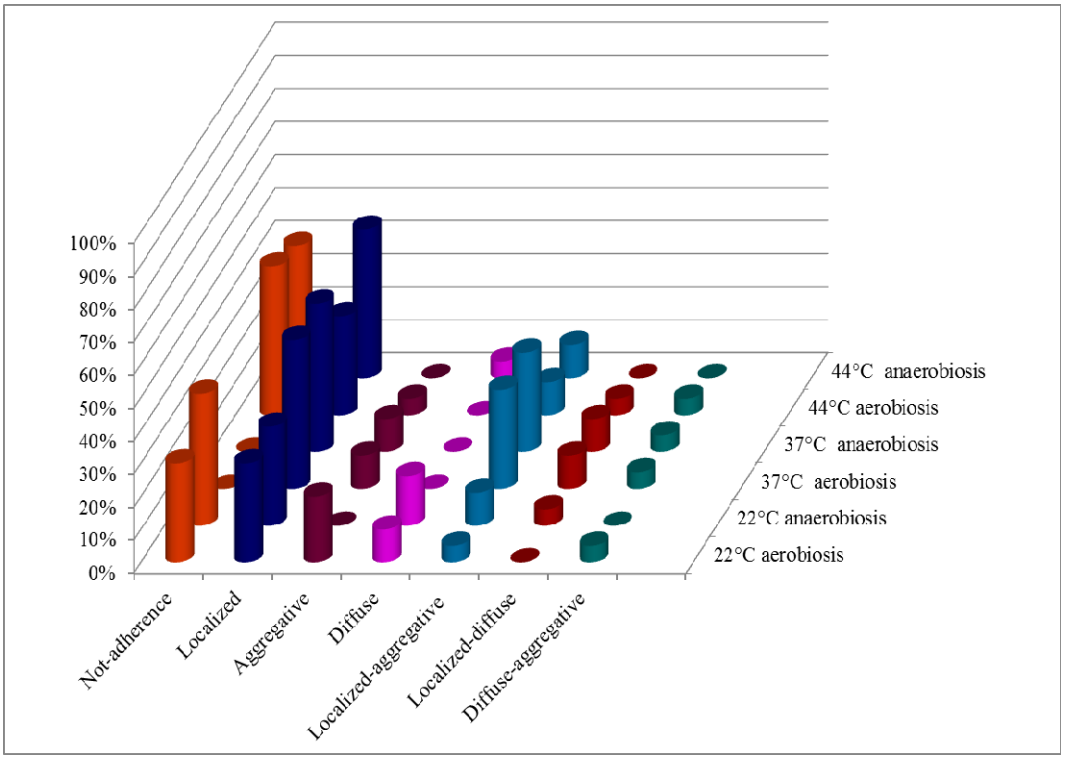

Fig. 4 - The influence of O_2 presence/absence on the bacterial adherence capacity to the HeLa cellular substratum (%), in *E. coli* strains isolated from sea water.

Each microbial species growth corresponds to an optimum pH, most developing at a neutral one (7.2 to 7.6), excepting *Vibrio cholerae* which develops at an alkaline pH (8.9 to 9.2), while yeasts and molds prefer the acid pH (Zarnea, 1994).

As shown in figure 5, the only bacteria cultivation condition reducing by only 15% the bacterial adherence capacity to the HeLa cellular substratum is the acid pH level in the culture medium. Both in acid and alkaline pH, all adherence patterns were expressed.

Maintaining the bacterial adherence capacity to the HeLa cellular substratum in the conditions of an alkaline pH reveals the capacity of *E. coli*strains isolated from sea water to initiate an infectious process, both in conditions of physiological and alkaline pH.

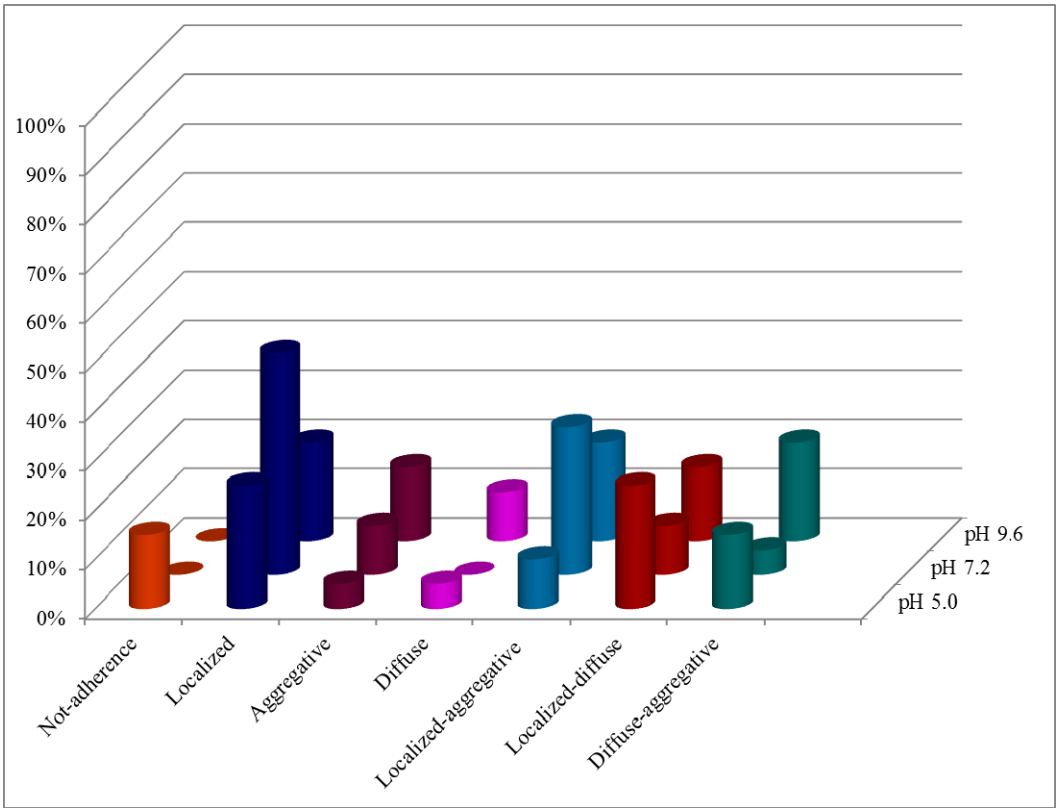

Fig. 5 - The influence of the culture medium's pH variation on the bacterial adherence capacity to the HeLa cellular substratum (%), in *E. coli* strains isolated from sea water.

This study has revealed the fact that the glucose concentration from the culture medium does not have significant influence on the bacterial adherence capacity to the HeLa cellular substratum. The patterns which were expressed in none of the two glucose concentrations were the aggregative and the diffuse-aggregative ones (fig. 6).

Low glucose concentration in the culture medium (1.5%) favors bacterial adherence to theHeLa cellular substratum (fig. 6).

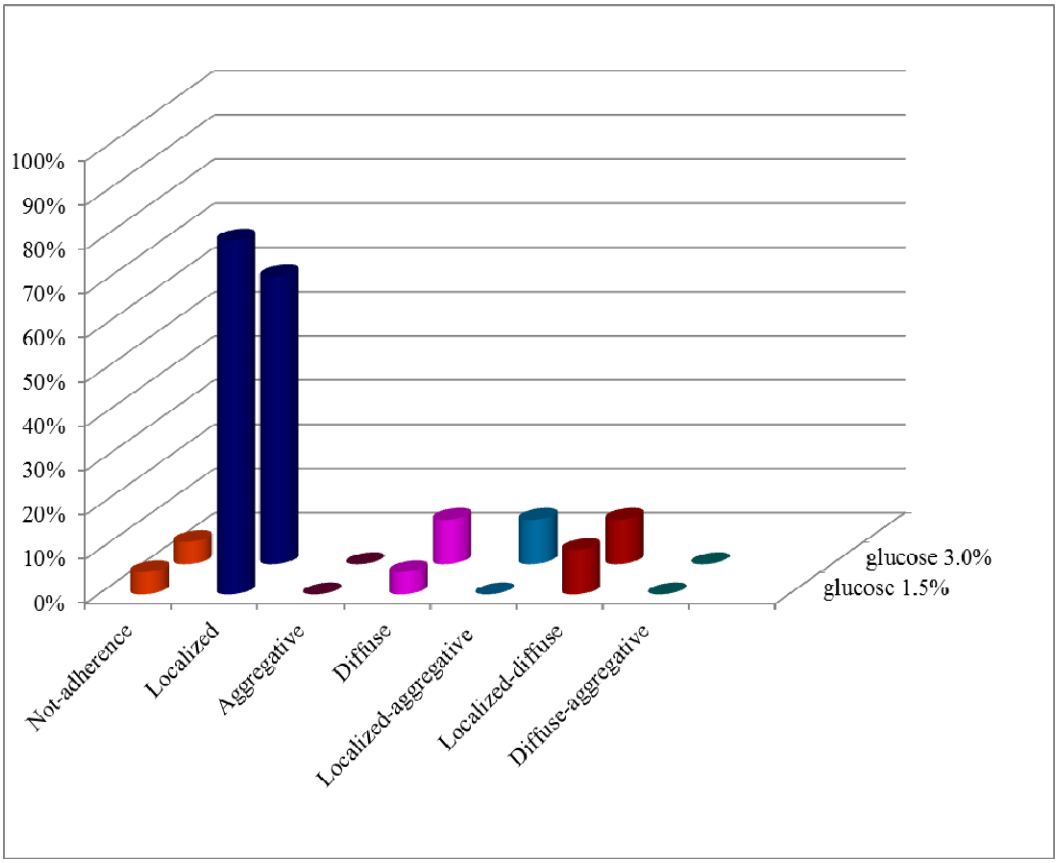

Fig. 6 - The influence of the glucose concentration from the culture medium on the bacterial adherence capacity to the HeLa cellular substratum (%), in *E. coli* strains isolated from sea water.

As this study has shown, the only NaCl concentration not influencing the bacterial adherence capacity to the HeLa cellular substratum is that of 0.5%, unlike the 4% and 5% concentrations, that have slightly influenced the bacterial adherence capacity to the cellular substratum, and the 2%, 6%, 7% and 10% which have significantly influenced this phenomenon. The most frequently expressed pattern was the localized one, regardless of the NaCl concentration in the culture medium. The NaCl concentrations allowing the expression of the most adherence patterns were the 0.5% and 5% ones, with 4 adherence patterns. The only salt concentration expressing the diffuse pattern was of 3%. Also, the only salt concentration expressing the diffuse-aggregative pattern was 0.5% (fig. 7).

In the present study, the *E. coli* strains isolated from the sea water have been investigated through the PCR technique for the presence of genes codifying proteins involved in adherence: *aggR*, transcriptional activator of genes for type I expression fimbrias in enteroaggregative*E. coli* (EaggEC), the genes for

afimbrialadhesins (*aafI / II*), and the genes involved in the synthesis of some toxins – the genes for thermostable enterotoxin (*EAST / 1*). All strains were negative for the presence of these genes.

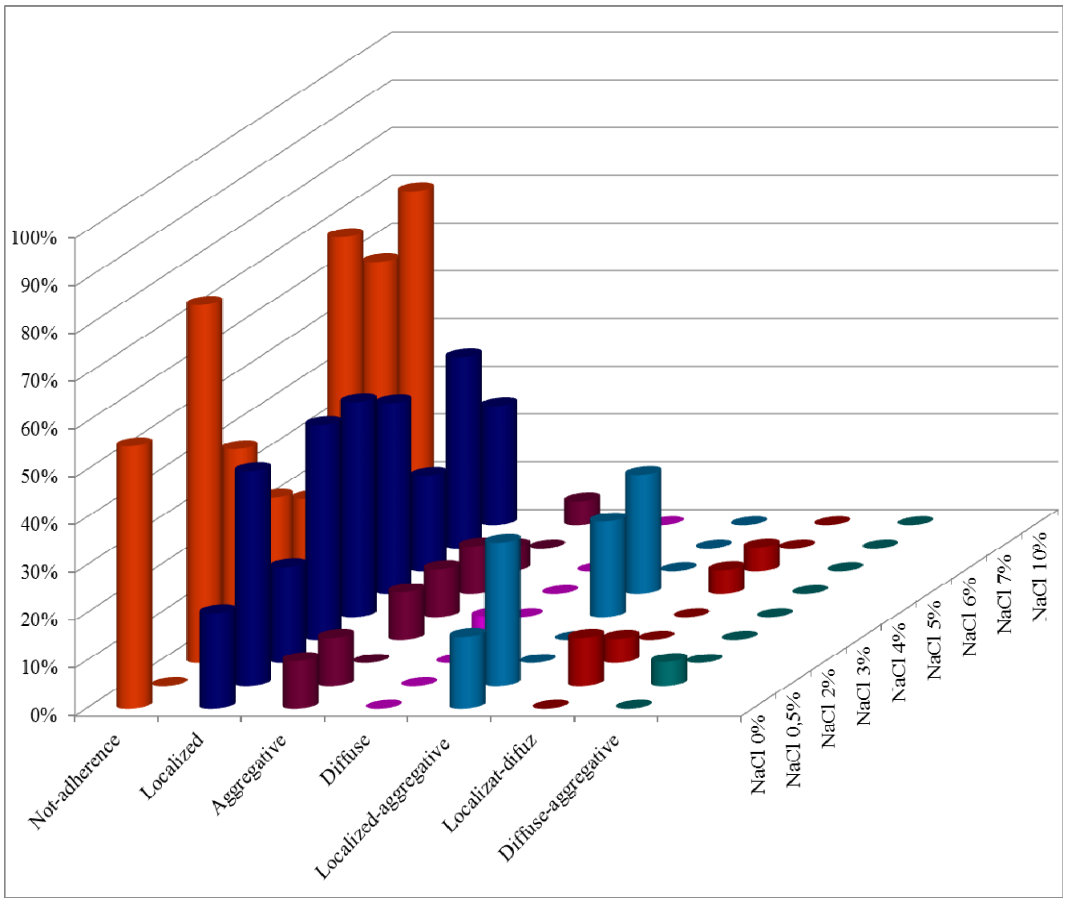

Fig. 7 - The influence of the NaCl concentration from the culture medium on the bacterial adherence capacity to the HeLa cellular substratum (%), in *E. coli* strains isolated from sea water.

Conclusions

The adherence capacity to the cellular substratum represented by HeLa cells was significantly influenced by the variation of the NaCl concentration in the culture medium.The mostfrequently expressed adherence pattern to the cellular substratum was the localized one.The maximum adherence capacity to the cellular substratum was highlighted by growing bacteria in liquid culture medium with alkaline pH of 9.6 and physiological conditions (pH 7.2 at 37°C, in aerobic conditions).The high levels of positivity in the adherence rates to biotic surfaces noticed in *Escherichia coli* strains isolated from the sea water plead for their potential to colonize human mucous surfaces or implanted prosthetic devices, thus being capable to initiate and develop an infectious process.

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