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Effect of different physico-mechanical factors upon the growth and expression of virulence in *Escherichia coli* strains isolated from drinking and sea water

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Abstract.
The purpose of the present study was to investigate the growth ability and expression of different virulence factors in 100 aquatic *Escherichia coli* strains isolated in Romania from drinking and sea water submitted to temperature and mechanical stress.
Material and methods. The isolated strains were investigated for cell-associated and soluble virulence factors, before and after exposure to freezing temperature for one month and respectively, after microbial culture centrifugation at 10000 rpm for 10 minutes.
Results. All tested strains produced haemolysins and lipase and exhibited also high positively levels for the production of DN-ase, esculinase and gelatinase. After the temperature and mechanical shock, 32% for the sea strains and only 2% of the drinking water strains were recovered and the virulence factors expression was drastically decreased.

Keywords: *Escherichia coli,* virulence factors, the temperature and mechanical shock.

1. Introduction

Contamination of surface waters by fecal pollution constitutes a serious environmental and public health threat. In large complex systems, fecal pollution can be introduced from multiple sources, including sewage overflows, agricultural runoff, and urban stormwater. Identifying and eliminating the source of contamination is not straightforward because assessment of fecal pollution generally relies on a limited number of surface water samples to measure fecal indicator organism densities (Byappanahalli et al., 2003; Gordon et al., 2002).

E. coli is a type of fecal coliform bacteria commonly found in the intestines of animals and humans. The presence of *E. coli* in water is a strong indication of recent sewage or animal waste contamination. During rainfalls, snow melts, or other types of precipitation, *E. coli* may be washed into creeks, rivers, streams, lakes, or ground water. When these waters are used as sources of drinking water and the water is not treated or inadequately treated, *E. coli* may end up in drinking water (Llopis et al., 2004).

Numerous studies provide evidence that *E. coli* can persist in the benthos environment and subsequently be detected in overlying surface waters (Torrella et al., 2003). Residual populations were reported in one study, where fecal coliform levels in wastewater subjected to low temperatures decrease rapidly but then stabilize to 1 to 10% of the initial population size. In addition, *E. coli* that has been isolated from septic tanks has been found to be less diverse and genetically distinct than strains of *E. coli* from the inhabitants of the households served by those systems.

Although most *E. coli* strains are harmless and live in the intestines of healthy humans and animals, this strain could exhibit powerful virulence factors and can cause severe illness with a large spectrum of etiologies.

The aim of the present study was to investigate the growth ability and expression of different virulence factors in aquatic *E. coli* strains submitted to temperature and mechanical stress.

MATERIAL AND METHODS

100 environmental *E. coli* isolated in Constanta, Romania from:drinking water (50) marine water (50).

The isolation and identification of these strains is 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 37°C, *E. coli* will develop yellow colonies on the membrane (**fig. 1**).

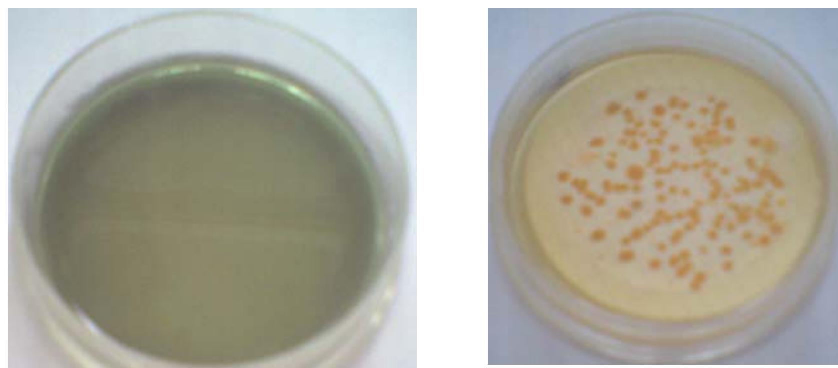

Fig. 1. The appearance of *E. coli* colonies on Lactose TTC medium (right) and the aspect of the sterile medium (left).

Oxidase and indole production test were performed additionally for the identification of *E. coli* strains. **(fig. 2**)

Fig. 2. The oxidase (left) and indole production (right) test.

Cell-associated virulence factors assay

The **bacterial ability to colonize the abiotic surface** was quantified by slime test (Christensen et al., 1982). The strains were cultivated in tubes with nutrient broth and incubated at 37°C for 24 h and thereafter the cultures tubes were emptied and stained with safranin alcoholic solution 1% for 30 minutes, washed three times with distilled water and left at room temperature for 24 h. The intensity of the red ring on the tube glass wall was noted with +, ++, +++, ++++.

The **adherence capacity to the biotic substrate** (HeLa cells) was investigated by using Cravioto method (adapted by us) (Lazăr, 2003). In this purpose 1 ml bacterial suspension prepared from a broth culture of 24 h 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.

Soluble enzymatic factors implicated in bacterial virulence

The **CAMP-like factor** was evidenced by streaking the tested strains at 8 mm distance from the β-haemolysis producing *Staphylococcus aureus* (ATTC 25923) on 5% sheep blood agar plates and incubated aerobically at 37°C for 24 h. The synergistic clear haemolysis noticed at the junction of the two spots areas, often with an arrow-like appearance, indicated the production of CAMP-like factor. The **plate hemolysis** was evidenced by streaking the tested strains on blood agar plates containing 5% (vol/vol) sheep blood in order to obtain isolated colonies. After incubation at 37°C for 24 h, the clear areas (total lysis of red blood cells) around the colonies were registered as positive reactions. For the investigation of **lipase production** the strains were spotted on Tween 80 agar as a substrate at a final

concentration of 1 % and were incubated at 37°C until 7 days. An opaque (precipitation) zone around the spot was registered as positive reaction. For **lecithinase production**, the cultures were spotted into 2.5% yolk agar and incubated at 37°C for 7 days. An opaque (precipitation) zone around the spot indicated the lecithinase production. **Decarboxilases: lysine-decarboxilase (LDC)** are active (efficient) in anaerobiose, at acid pH and catalyzes in presence of phosphate pyridoxal coenzyme, the ornitine (dyamino-monocarboxilic aminoacids) and LDC reaction involves the specific dyamines appearance. In practice, the ODC, LDC and ADH detection is made by evidencing the pH medium variation. The glucose fermentation by bacteria with fermentative metabolism requires culture medium acidity and the medium color turns in to yellow. If the bacteria don't express LDC in their enzymatic equipment, the medium remains yellow and, in presence of these enzymes, there it will be a secondary realkalinisation of the medium due to dyamine-cadaverine production and the medium color turns again into purple. The **DN-ase production** was studied on DNA supplemented medium. The strains were spotted and after incubation at 37°C for 24 h, a drop of HCl 1N solution was added upon the spotted cultures; a clearing zone around the culture was interpreted as positive reaction. The **siderophores production** was tested by esculyin hydrolysis reaction. In this purpose the strains have been streaked on esculin and Fe^{3+} citrate ($FeC_6H_5O_7$) containing medium and the esculetol (acting as siderophore production) was indicated by the occurrence of a black color, after incubation at 37°C until 5 days. The **caseinase activity** was determined using 15% soluble casein agar as substrate. The strains were spotted and after incubation at 37°C for 24 h, a clearing zone surrounding the growth indicated casein proteolysis. The **mucinase production** was determined using pig stomach mucine (final concentration of 1%) incorporated in brain heart agar with 2% NaCl. The strains were spotted and incubated at 35°C for 48 h; the enzyme activity was noticed by the presence of a clear area around the culture spot; the clear area became more evident when some Lugol drops were poured upon. The **amylase production** was tested on agar medium supplemented with 10% starch. The strains were stubbed and incubated at 37°C for 24 h, starch hydrolysis was registered by the presence of a clear area around the culture spot (Burke et al., 2001, Centre de l'Enseignement de l'Institut Pasteur de Paris., 2000; Figura and Guglielmetti**,** 1987; Lenette et al., 1980; Wiggins et al., 2001.).

For gelatinase production the **Frazier** technique consisting in the spot inoculation of nutritive agar with gelatin and incubation for 1, 2, 3 days at 37°C, followed by the gelatin precipitation with sublimate.

RESULTS AND DISCUSSION

After the temperature and mechanical shock, 32% for the sea strains and only 2% of the drinking water strains were recovered demonstrating the role of the natural environment in the development of microbial resistance to stress factors.

Results – virulence hallmarks

Adherence to abiotic substratum

Before the physico-mechanical stress action all tested strains exhibited the adherence capacity to the abiotic substratum. After the thermal and mechanical stress, drinking water strains lost entirely this ability, while those isolated from the sea water preserved this capacity in 84% of strains (**fig. 3**).

Fig. 3 The slime test before (left) and after (right) physico-mechanical factors action.

Adherence to biotic substratum

Regarding adherence to the biotic substrate and the influence of physical factors, all the strains isolated from seawater were adherent to the biotic (HeLa cells) but after heat and mechanical treatment the adherence was reduced to 63%, mostly with diffuse adhesion pattern (67%), followed by aggregative (16%) and localized (8%) ones (**fig. 4**).

In case of drinking water strains the mechanical and physical stress factors did not influence the rates of adherence.

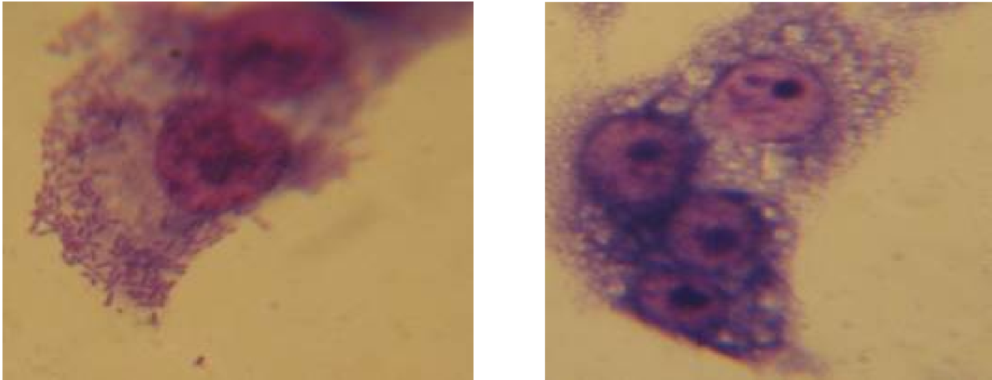

Fig. 4. Optical microscopy image of the HeLa cells infected with *E. coli* strains isolated from sea water, adhering to cells in the culture before (left) and after (right) physico-mechanical factors action (Giemsa, x100 coloration).

Soluble enzymatic virulence factors

All tested strains produced LDC before and after thermal stress (**fig. 5**).

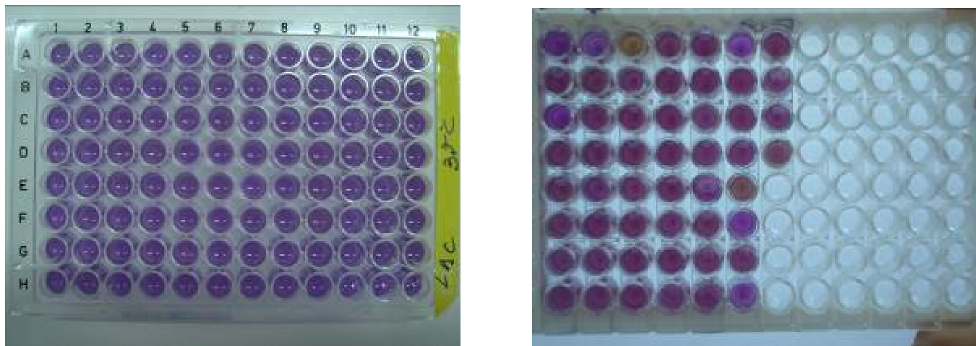

Fig. 5 Production of the LDC, before (left) and after (right) physico-mechanical factors action.

The mechanical and physical factors influenced the expression of virulence factors (**fig. 6, 7**).

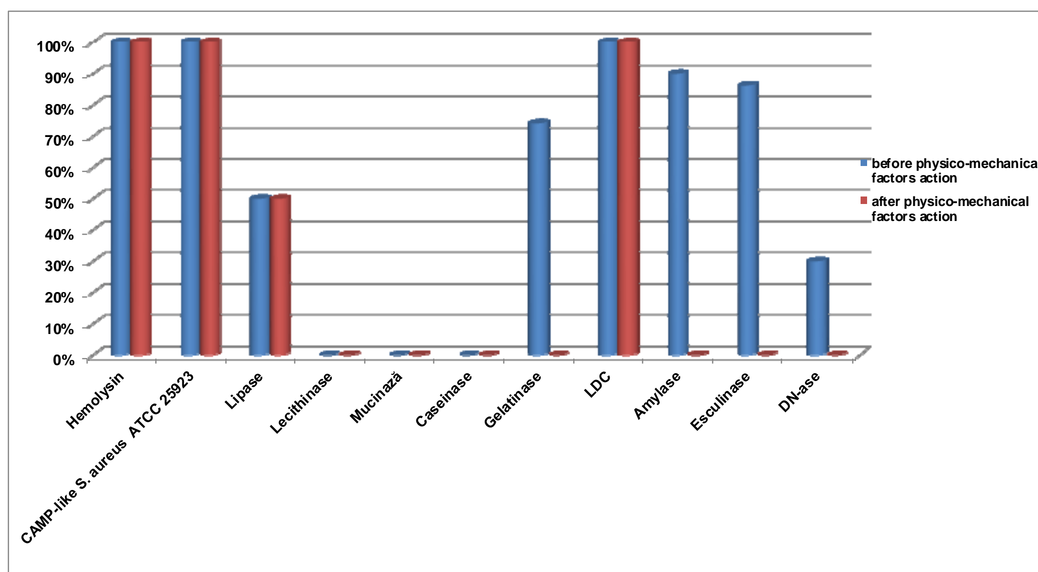

Fig. 6. The level of positivity of different soluble virulence before and after physico-mechanical factors action *Escherichia coli* strains isolated from drinking water.

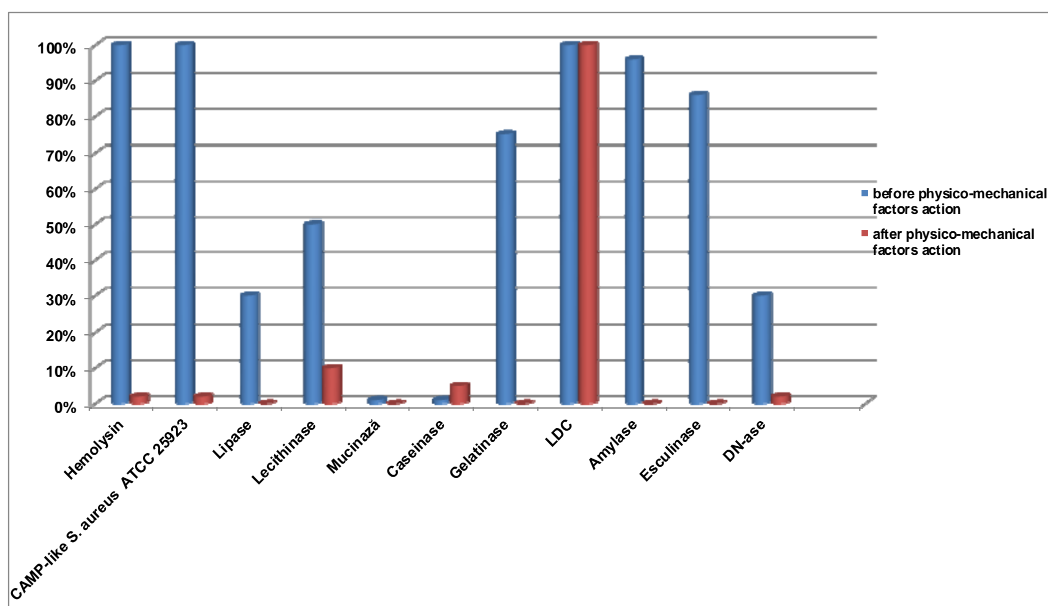

Fig. 7. The level of positivity of different soluble virulence before and after physico-mechanical factors action *Escherichia coli* strains isolated from sea water .

The temperature and mechanical shock drastically inhibited the expression of haemolysins, CAMP-like factor, lipase, gelatinase, amylase and DN-ase (**fig. 8, 9, 10**).

Fig. 8. Production of the haemolysis, before (left) and after (right) physico-mechanical factors action.

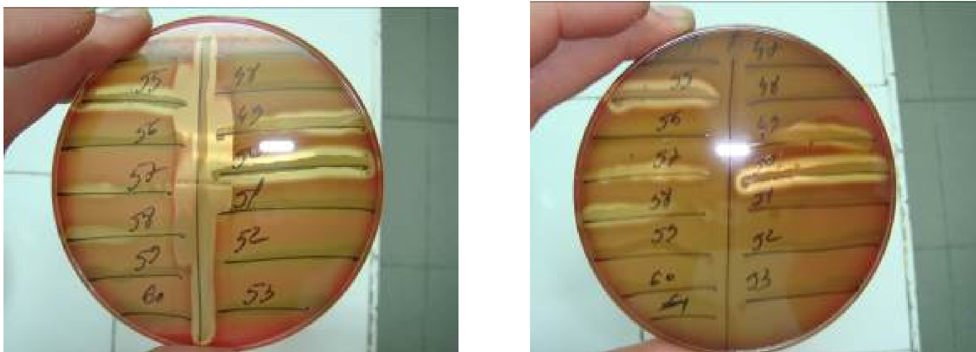

Fig. 9. Production of the CAMP factor before (left) and after (right) physico-mechanical factors action.

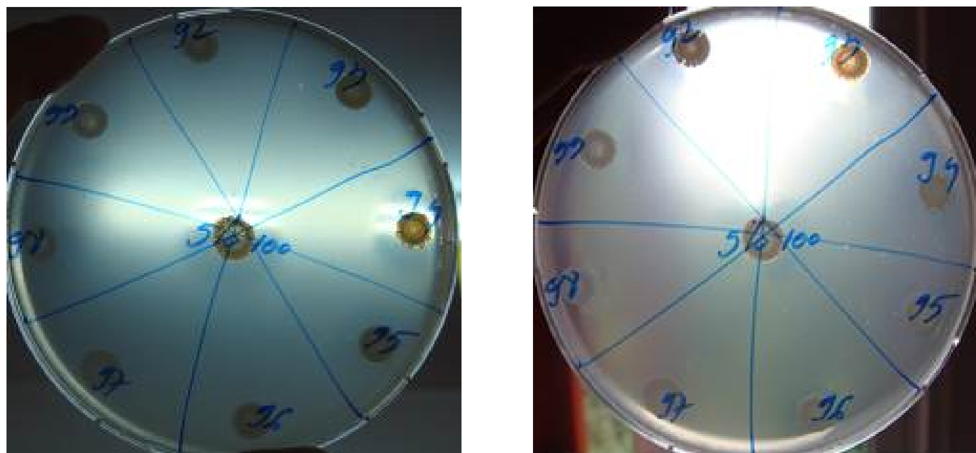

Fig. 10. Production of the amylase before (left) and after (right) physico-mechanical factors action.

CONCLUSION

The high positivity levels of adherence to abiotic and biotic surfaces is pleading for the potential ability of these strains to colonize the human mucosal surfaces and thus to initiate and develop an infectious process, sustained by the secretion of soluble enzymes, such as lipases and glucidases.

The great majority of marine and drinking water strains proved to be positive for the production of lysinedecarboxilase which is responsible for the production of a biogenic toxic dyamine named cadaverine.

Concerning other biochemical markers which are supporting the implication in the pathogenicity of *Escherichia coli,* our results are pleading for the existence of a pathogenic potential in *Escherichia coli* aquatic strains, which could colonize the cellular tissues and thereafter cause invasive infections, by the secretion of haemolysins and lipases (acting as pore-forming toxins) and of tissue degrading enzymes, such as gelatinases.

The results of the present study have shown that aquatic medium signifies an appropriate ecological system for the existence and maintenance of a complex reservoir of virulence factors with high risk for human host colonization and implications in the human health.

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