Virulence, pathogenicity, antibiotic resistance and plasmid profile of *Escherichia coli* strains isolated from drinking and recreational waters

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Abstract

The aim of this study was to investigate the antibioresistance profile and the virulence and pathogenicity hallmarks of Escherichia coli acquatic strains. Material and methods. 100 environmental Escherichia coli were isolated from drinking water (50) and marine water (50) in Constanta, Romania. They were identified both by biochemical and serological tests. Both the disc diffusion susceptibility test and the microplate dilution technique were used to investigate the antibiotic resistance profile of these bacteria. The rapid test to nitrocephine and isoelectrofocusing techniques were used for confirming the presence and type of beta-lactamases. The analysis of plasmidial DNA was performed with a Wizard extraction kit. The virulence tested features were: adherence and invasion capacity on HeLa cells by Cravioto adapted method, adherence on inert substrata quantified by slime test, production of extracellular enzymes and exotoxins (haemolysins and other pore-forming toxins, amylase, mucinase, gelatinase, caseinase, aesculin hydrolysis). Results and discussion. Results from both antibiosusceptibility assays were generally in good agreement. The tested strains, irrespective of the source of isolation, exhibited resistance to ampicillin, ticarcillin, tetracyclines and sulphametoxazole and were susceptible to all other tested antibiotics. 21 strains exhibiting resistance to beta-lactam antibiotics (7 isolated from drinking and 14 from marine waters) proved to be positive for the presence of beta-lactamases when tested by nitrocephine rapid test. The beta-lactamases presence in the tested strains was confirmed by isoelectrofocusing, the enzymes exhibiting an isoelectric point ranging from 7,5 to 9,6. 10% of the total of drinking water strains exhibited 1 to 3 plasmids, while marine strains only 1 plasmid. As far as the virulence hallmarks harboured by the tested strains is concerned, 90% of the strains isolated from drinking water exhibited high capacity of adherence to the cellular substrate (adherence indexes of 85-100% with localized, aggregative and diffuse patterns). This proves the potential of these strains to colonize animal and human tissues and initiate an infectious process as compared to the marine strains showing low adherence potential. All Escherichia coli strains showed colonization ability of the inert substrate as demonstrated by the high positivity rate of slime test. All tested strains produced lipase, which could act as pore-forming toxin in case of tissue colonization. Our results point out the importance of detecting specific virulence factors before incriminating water as a source of human diseases.

Keywords: *Escherichia coli,* aquatic strains, virulence, antibiotic resistance, plasmid profile, strains isolated from

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 storm water. 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 [1, 2, 9]. *Escherichia coli* are 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 [5]. Numerous studies provide evidence that *E. coli* can persist in the benthos environment and subsequently be detected in overlying surface waters. 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 antibioresistance profile and the virulence and pathogenicity hallmarks of *Escherichia coli* acquatic strains isolated from drinking and sea waters.

Material and methods

Bacterial strains. In this study, we analyzed 100 environmental *E. coli* strains isolated in Constanta, Romania from drinking water (50) and marine water (50) respectively. The isolation and identification of these strains is

based on filter membrane method, according to SR ISO 9308-1 2000 [7]. 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 on 47 mm diameter Petri plates. After 48 hours incubation at 37°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.

Antibiotic susceptibility profiles of the strains were determined by the disc diffusion method. Plates of Mueller-Hinton agar were inoculated with a bacterial suspension equivalent to a 0.5 McFarland standard and incubated aerobically at 37°C for 18 h. Results were expressed as susceptible or resistant according to the criteria adopted by the NCCLS/CLSI (2006), using the following antibiotics: amoxicillin (AMX), gentamycin (GEN), tobramycin (TOB), ciprofloxacin (CIP), cefotaxime (CTX), cephtazidime (CAZ), imipeneme (IMP), aztreonam (AZT), nalidixic acid (NAL), ofloxacin (OFX), netilmicin (NET), tetracycline (TCY), piperacillin (PIP) and piperacillin plus tazobactam (PTZ) (Oxoid, Basingstoke, Hampshire, England disks).

Confirmation of betalactamases production was performed by nitrocephine chromogenic test, double disk diffusion test [6, 8] and MICs for ß-lactams determined by using nutrient broth microdillution method and E-test ESBL strips (AB Biodisk, Dalvägen, Sweden) [6, 8].

The molecular approach of the antiboresistance was performed by isoelectrofocusing and the a**nalysis of plasmidial DNA** (performed using Wizard extraction kit, Promega) [6, 7, 8].

Cell-associated virulence factors assay

The **adherence capacity to the biotic substrate** (HeLa cells) was investigated by using the Cravioto method (adapted by us). 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 37^0C, 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.

The **bacterial ability to colonize the abiotic surface** was quantified by slime test. The strains were cultivated in tubes with nutrient broth and incubated at 37^0C for 24 h and thereafter the cultures tubes were further 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 +, ++, +++, ++++.

Soluble enzymatic factors implicated in bacterial virulence

The **CAMP-like factor** was evidenced by streaking the tested strains at 8 mm distance from the beta-haemolysis producing *Staphylococcus aureus* (ATTC 25923) on 5% sheep blood agar plates and incubated aerobically at 37^0 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 [3, 4]. 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 37^0C for 24 h, the clear areas (total lysis of red blood cells) around the colonies were registered as positive reactions [3, 4]. 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 37^0C until 7 days. An opaque (precipitation) zone around zone around the spot was registered as positive reaction [3, 4]. For **lecithinase production**, the cultures were spotted into 2,5% yolk agar and incubated at 37^0C for 7 days. An opaque (precipitation) zone around the spot indicated the lecithinase production [3, 4]. The **DN-ase production** was studied on DNA supplemented medium. The strains were spotted and after incubation at 37^0 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 [3, 4]. The **caseinase activity** was determined using 15% soluble casein agar as substrate. The strains were spotted and after incubation at 37^0C for 24 h, a clearing zone surrounding the growth indicated casein proteolysis [3, 4]. 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 until 35^0C 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 [3, 4, 10].

The **amylase production** was tested on 10% starch supplemented agar medium. The strains were stubbed and incubated at 37 0 C for 24 h, starch hydrolysis was registered by the presence of a clear area around the culture spot [3, 4].

Results and discussion

Antibiotic susceptibility data. The aquatic *Escherichia coli* strains were generally susceptible to the majority of tested antibiotics. Irrespective of the source of isolation the tested strains exhibited resistance to ampicillin and tetracyclines. The sea strains exhibited an extended spectrum of antibioresistance, exhibiting simultaneous resistance to ampicillin, tetracyclines, ticarcillin, sulphametoxazole, pefloxacin and nalidixic acid (Fig.1).

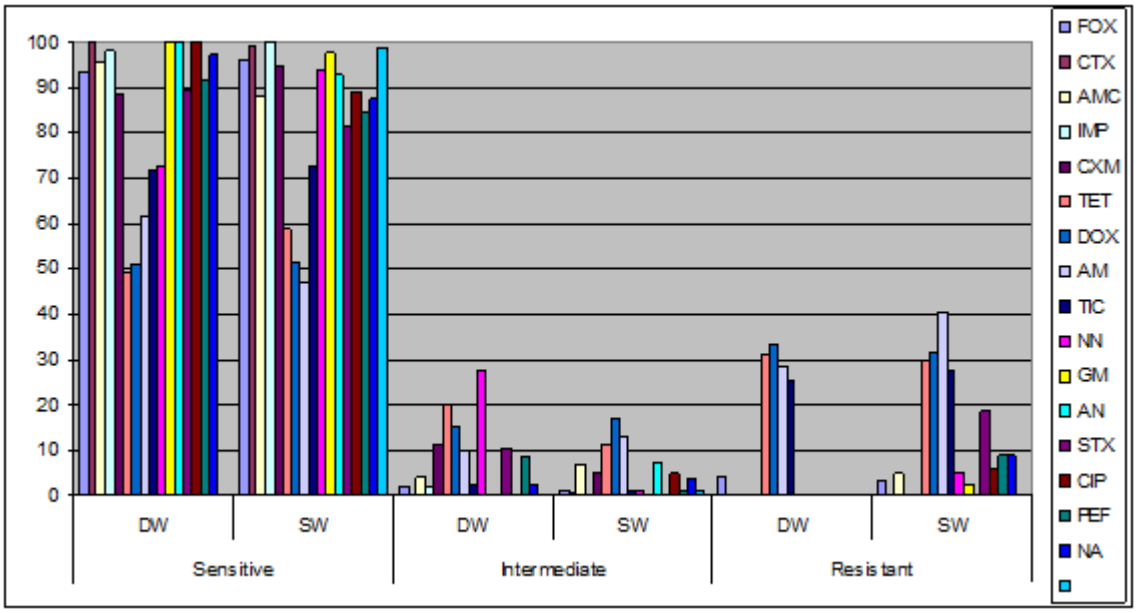

Figure 1. The comparative levels of antibiotic resistance in DW and SW strains

Beta-lactamase *screening* and confirmation tests. 21 (14.66%) aquatic strains exhibiting resistance to beta-lactam antibiotics (7 isolated from drinking and 14 from sea waters) proved to be positive for the presence of beta-lactamases when tested by nitrocephine rapid test. The beta-lactamases presence in the tested strains was confirmed by isoelectrofocusing, the enzymes exhibiting an isoelectric point ranging from 7,5 to 9,6.
Plasmid DNA isolation was performed on 21 strains (7 from drinking water and 14 from sea) exhibiting multiple resistance markers. The presence of plasmid DNA was revealed in 33% of the tested strains. The isolated plasmids (1 to 3 strains) exhibited molecular weights ranging from 1.00 bp to 23 kbp (Fig. 2).

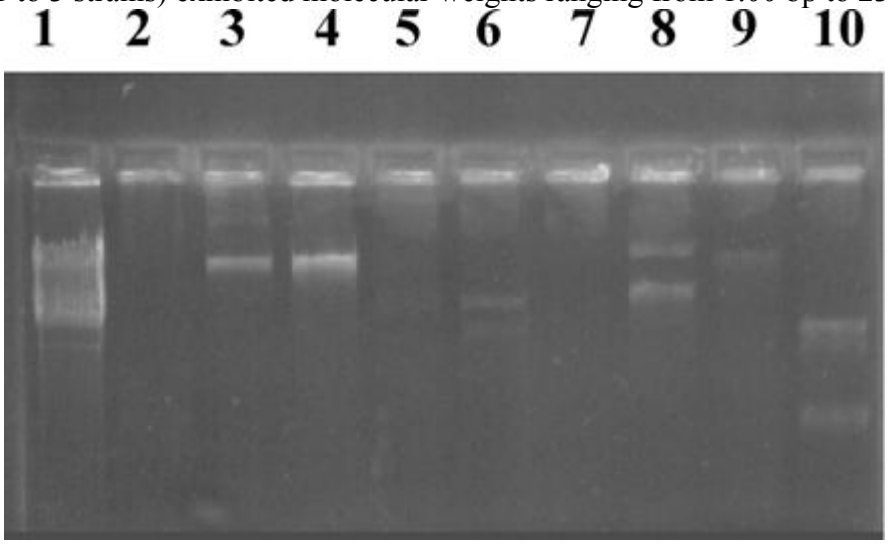

Figure 2. Gel electrophoresis of plasmid DNA. The well contain from left to right: 1. *E. coli* DW 1257 -06; 2. *E. coli* DW 679-06; 3. *E. coli* DW 320-06; 4. *E.coli* SW 577-07; 5. *E. coli* DW 159-06; 6 *E. coli* DW 173-06; 7. *E. coli* DW 684-06; 8. *E. coli* DW 181-05; 9. *E. coli* SW 583-07; 10. DNA/HindIII

As far as the adherence to the biotic substratum 90% of the strains isolated from drinking water is concerned, it exhibited high capacity of adherence to the cellular substrate (adherence indexes of 85-100% with localized, aggregative and diffuse patterns) demonstrating the potential of these strains to colonize the animal and human tissues and to initiate an infectious process. The marine strains exhibited very low adherence capacity to the cellular substrate. All tested strains *E.coli* strains showed high colonization ability of the inert substrate as demonstrated by the high positivity rate of slime test. The tested strains scarcely expressed soluble enzymatic virulence factors, i.e. the DW strains produced lipase, which could act as pore-forming toxin in case of tissue colonization and both DW and SW strains produced amylase, which could be implicated either in survival of strains in the external environment, or in the colonization.

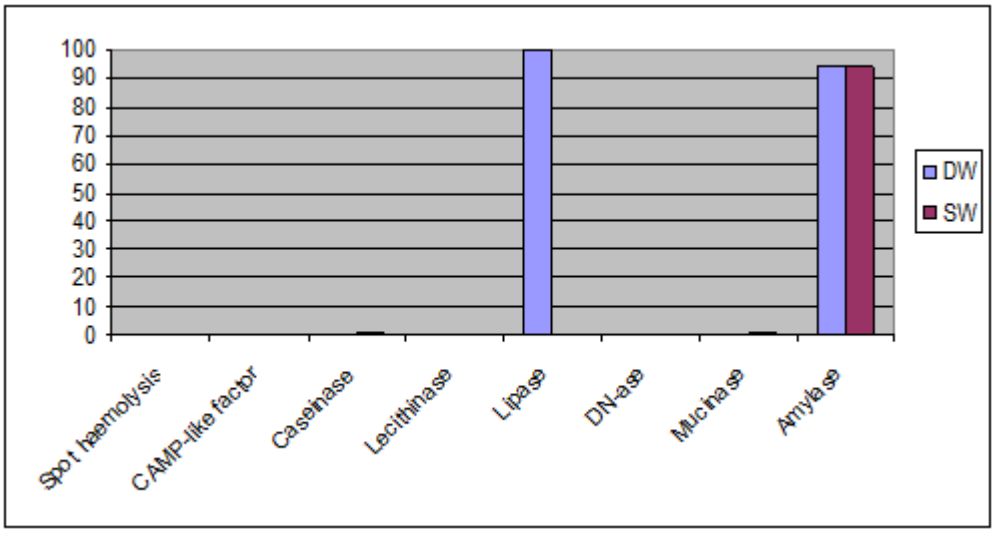

Figure 3. The comparative expression level of different soluble virulence factor in DW and SW *E. coli* strains

Conclusion

Our study revealed that *Escherichia coli* strains isolated from sea waters exhibit a higher level of multiple antibioresistance than drinking water strains, probably due to the existence of unspecific efflux pumps required for the survival and adaptation to hypersaline conditions. 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 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 antibioresistance and virulence factors with high risk for human host colonization and implications in the human health.

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