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Phenotypic and genetic investigation of virulence and antibioresistance hallmarks in *Escherichia coli* strains isolated from Black Sea water on Romanian coast

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ABSTRACT

The aim of this study was to investigate by phenotypic and molecular tools the antibiotic resistance profile and the virulence markers of 100 environmental *Escherichia coli* strains isolated from marine water in Constanta, Romania. The antibiosusceptibility testings were performed by disk diffusion method (CLSI, 201, including the phenotypic screening of beta-lactamases. The presence of different antibiotic resistance markers was correlated with the plasmidial pattern of the analyzed strains. Eleven (11) virulence factors were tested by using specific culture media. PCR was performed for the following antibiotic resistance and virulence genes: *sul 1, 2, 3, aggR, EAST-1,* AAFI/II. The tested strains exhibited high susceptibility to imipenem, ceftriaxone, cefoxitin and nitrofurantoin (99%), tobramycin (98%), cephtazidime (97%), gentamycin and amikacin (96%), ciprofloxacin (93%), nalidixic acid and chloramphenicole (92%) sulphametoxazole (89%) and high resistance levels to ampicillin (94%). The tested strains exhibited between 1 and 8 antibioresistance markers, the most frequent associations being: MP+SXT (10%), AMP+C (9%), AMP+CIP (8%) and AMP+NA (7%). Morevoer, the tested strains exhibited tolerance to heavy metals, i.e. to Zn (72%), Mn (98%), Cu (98%), Co (95%), and Ni (99%). 43,6% strains exhibiting resistance to beta-lactam antibiotics proved to be positive for the presence of beta-lactamases when tested by nitrocephine rapid test. The synergy test was negative for all tested strains. 25% of the tested strains exhibited at least one plasmid with variable molecular weights. Concerning the virulence hallmarks harboured by the sea water strains, 44% exhibited capacity of adherence to the cellular substrate (adherence indexes of 30% with localized, aggregative and diffuse patterns) and inert substrata (60%). Unexpectedely, an important number of strains (70.37%) also exhibited invasion ability of HeLa cells demonstrating the potential of these strains to colonize the animal and human tissues and to initiate an infectious process.The tested strains produced mucinase (100%), lysin-decarboxilase (93%), aesculin hydrolysis (67%) and β-hemolysins (6%).

Keywords: *Esherichia coli, Black Sea, antibioresistance, virulence factors*

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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 [1,2]. *Escherichia 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 [3]. 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 [4]. In addition, *E. coli* that has been isolated from septic tanks was less diverse and genetically distinct than strains of *E. coli* from the inhabitants of the households served by those systems [5]. Although most *E. coli* strains are harmless and live in the intestines of healthy humans and animals, these strains could exhibit virulence features and can cause severe illnessess, with a large spectrum of clinical symptoms. The aim of the present study was to investigate the antibioresistance profiles and the virulence and pathogenicity hallmarks of *Escherichia coli* acquatic strains isolated from sea waters.

2. EXPERIMENTAL SECTION

100 environmental *Escherichia coli* isolated in Constanta, Romania from sea water. The isolation and identification of these strains was based on filter membrane method, according to SR ISO 9308-1 2000 [6]. 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, *Escherichia coli* will develop yellow colonies on the membrane. Oxidase and indole production test were performed additionally for the identification of *Escherichia coli* strains.

2.1. 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 [7]. The results were expressed as susceptible or resistant according to the criteria adopted by the NCCLS/CLSI (2011), using the following **a**ntibiotics (Oxoid, Basingstoke, Hampshire, England disks) tested for *Enterobacteria*: ampicilin (AMP), ceftazidim (CAZ), imipenem (IMP), ceftriaxone (CRO), cefoxitin (FOX), ciprofloxacin (CIP), nalidixic acid (NA), gentamycin (G), amikacin (AK/AN), tobramycin (NN), cotrimoxazole (SXT), cloramfenicol (C), nitrofurantoin (F/M). The tested strains have been also tested for their resistance to heavy metals: Zn, Mn, Cu, Co and Ni.

Confirmation of beta-lactamase production was performed by nitrocephine chromogenic test, double disk diffusion test and MICs for ß-lactams determined by using nutrient broth microdillution method and E-test ESBL strips (AB Biodisk, Dalvägen, Sweden). Plasmid DNA extraction was performed using Wizard extraction kit (Promega) [8,9].

2.2. The adherence capacity to the biotic substrate (HeLa cells) was investigated by using Cravioto' adapted method. In this purpose 1 ml bacterial suspension prepared from a broth culture of 24 h was inoculated on an 80% confluent cellular layer of HeLa-2 cells. After an incubation of 2 h at 37ºC, the bacterial suspension was discarded and the cell culture washed and Giemsa stained [10]. The adhesion was microscopically examined for the identification of the adhesion patterns (i.e. diffuse, localized and aggregative) and for the qualitative assay of adherence ability (+, ++, +++, ++++).

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 37C 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 +, ++, +++, ++++.

2.3. Screening for soluble enzymatic factors implicated in bacterial virulence

2.3.1. CAMP-like factor: the tested strains were streaked at 8 mm distance from the beta-haemolysis producing *Staphylococcus aureus* (ATTC 25923) strain on 5% sheep blood agar plates and incubated aerobically at 37ºC for 24h. 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 [11,12].

2.3.2. Plate hemolysis: the strains were streaked on blood agar plates containing 5% (vol/vol) sheep blood in order to obtain isolated colonies. After incubation 24 h at 37ºC, the clear areas (total lysis of red blood cells) around the colonies were registered as positive reactions.

2.3.3. Lipase production: cultures were spotted on Tween 80 agar as a substrate at a final concentration of 1% and were incubated at 37ºC until 7 days. An opaque (precipitation) zone around the spot was registered as positive reaction.

2.3.4. Lecithinase production: cultures were spotted into 2.5% yolk agar and incubated at 37ºC until 7 days. An opaque (precipitation) zone around the spot indicated the lecithinase production.

2.3.5. Lysin decarboxilase production was evidenced on MILF medium, the positive reaction being indicated by the occurrence of purple color.

2.3.6. Gelatinase production was evidenced by Frazier method, consisting in spotting the bacterial culture on gelatine agar medium and further incubation for 1 to 3 days at 37^{o}C, the positive reaction being observed by the occurrence of a clear zone aroud the culture spot after adding sublimate.

2.3.7. DN-ase production was studied on DNA medium. The strains were spotted and after incubation at 37ºC 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 [13].

2.3.8. Caseinase activity was determined using 15% soluble casein agar as substrate. The strains were spotted and after incubation at 37ºC for 24 h, a clearing zone surrounding the growth indicated casein proteolysis.

2.3.9. Mucinase production was determined using pig stomach mucine (final concentration of 1%) in brain heart agar with 2% NaCl. The strains were spotted and incubated until 48 h at 35ºC, the enzyme activity being 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 [14].

2.3.10. Amylase production was tested on 10% starch supplemented agar medium. The strains were stubbed and incubated at 37ºC for 24 h, starch hydrolysis was registered by the presence of a clear area around the culture spot.

2.3.11. Esculine hydrolysis produces the esculetol, acting as iron chelating agent. In the presence of Fe^{3+} citrate ($FeC_6H_5O_7$), the esculetol generates the formation of a black precipitate accumulated in the culture medium.

3. RESULTS SECTION

The aquatic *Escherichia coli* strains were generally susceptible to the majority of tested antibiotics, and exhibited constitutive resistance to ampicillin. Although the resistance rates were very low, the resistant strains exhibited simultaneous resistance to ampicillin, tetracyclines, ticarcillin, sulphametoxazole, pefloxacin and nalidixic acid (Figure 1). The most frequent associations of resistannce markers were: AMP+SXT (10%), AMP+C (9%), AMP+CIP (8%) and AMP+NA (7%) (Figure 4).

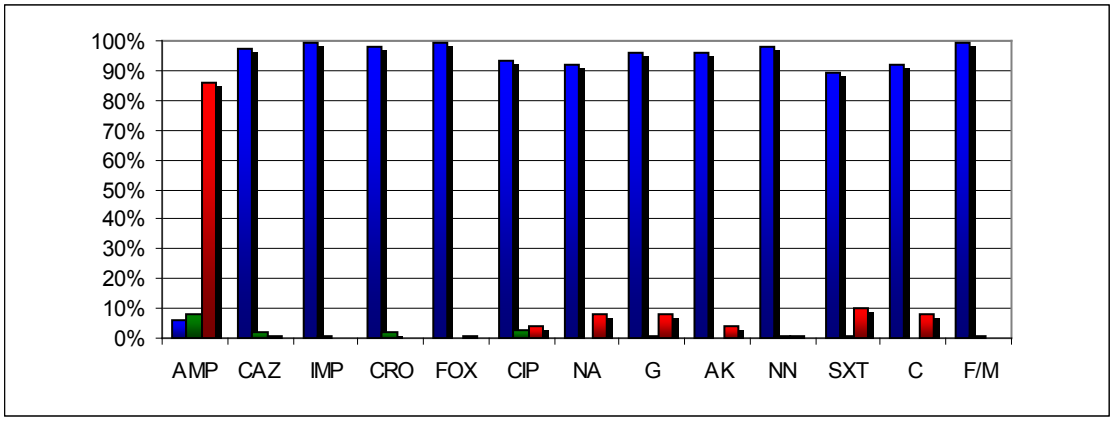

Figure 1: Levels of RIS (%) in the tested strains (blue- susceptibility-R, red- resistant-R, green- intermediate-I)

The multiple resistance to antibiotics could be plasmid-encoded, having in view that the resistant strains exhibited at least one plasmid with variable molecular weights (**Fig.2**).

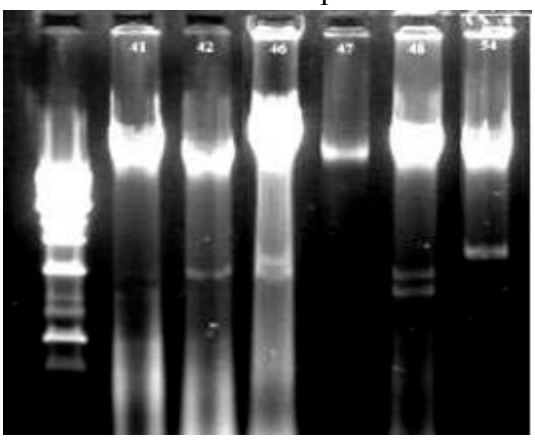

Figure 2: Total DNA migration profile in 7% agarose g

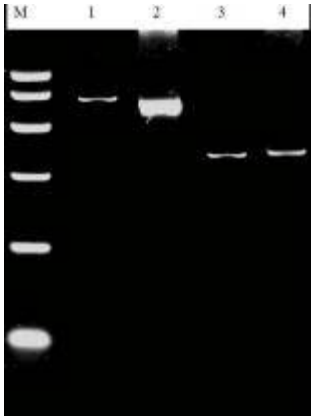

Figure 3: PCR amplicons migration profile in 1.5% agarose gel. Lane M, molecular mass markers: lanes 1, 2 –positive samples amplified with sul 2 primers; lanes 3, 4 – positive samples amplified with sul2 primer

All sulphonamide resistant *E. coli* isolates were investigated for the presence of *sul1*, *sul2* and *sul3* genes by PCR. The *sul1* gene was detected in 55% of the sulphonamide resistant isolates. The *sul2* gene was detected in 22% of isolates. None of the isolates were PCR-positive for *sul3*. (Figure 3). 43,6% strains exhibiting resistance to beta-lactam antibiotics proved to be positive for the presence of beta-lactamases when tested by nitrocephine rapid test (Figure 4).

Figure 4: Appearance of beta-lactamase positive strains in rapid chromogenic test to nitrocephine

Concerning the resistance to heavy metals, the majority of the tested strains proved to be resistant to heavy metals (Figure 5).

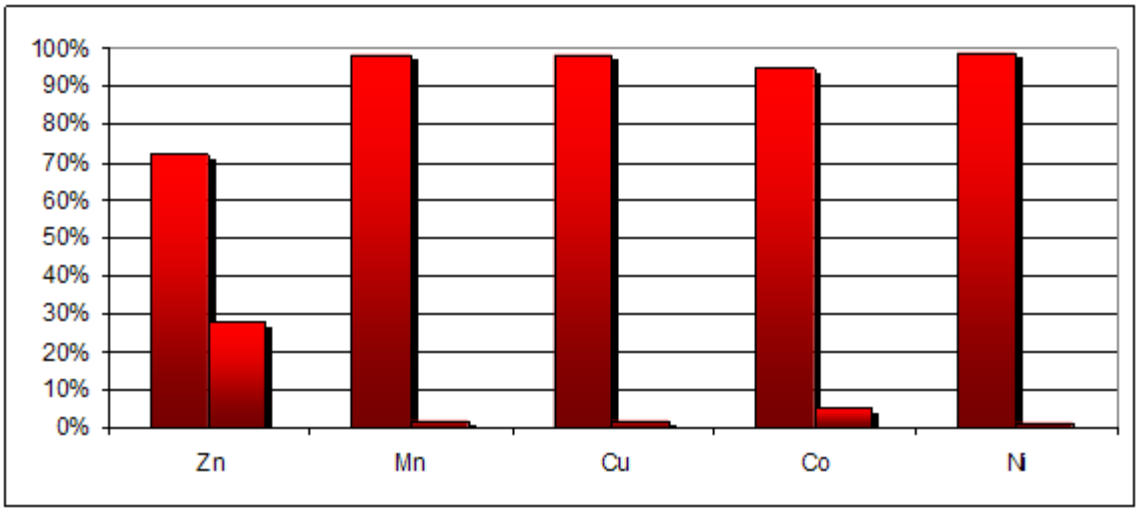

Figure 5: RS levels (%) of *E. coli* strains to heavy metals (red- resistance- R, blue-susceptibility-S)

Concerning the expression of different virulence hallmarks, the slime test revealed that 60% of the tested strains exhibited the ability to adhere to the inert substratum and 44% to the cellular substrate, all three adherece patterns (localized, aggregative and diffuse patterns) described for *E. coli* being present (Figure 6). The aggregative adherence pattern being the predominant one, the respective strains have been tested for the presence of molecular markers of entero-aggregative *E. coli* pathovar. All strains were negative for the transcriptional activator for EAggEC aggregative adherence fimbria I expression (*aggR*), the aggregative adherence fimbriae I (AAFI/II), and *E. coli* heat-stable enterotoxin *1 (EAST/1)*. Unexpectedely, an important number of strains (70.37%) also exhibited invasion ability of HeLa cells demonstrating the potential of these strains to colonize the animal and human tissues and to initiate an infectious process.

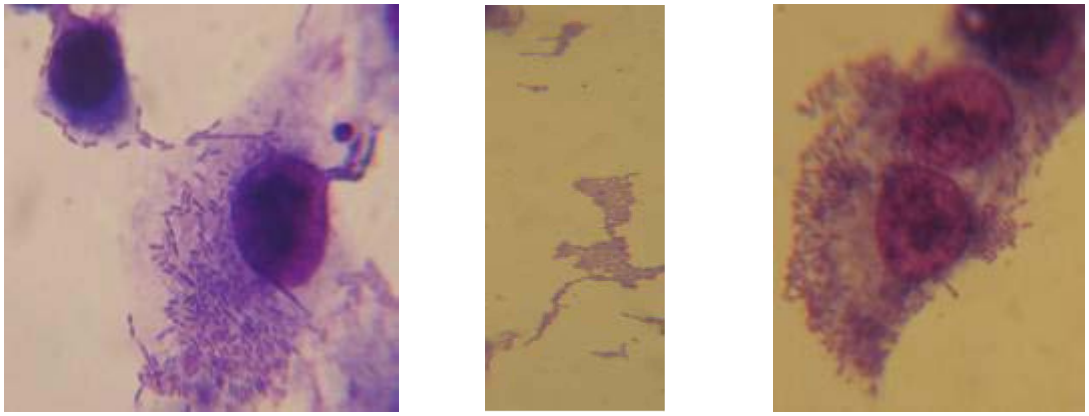

Figure 6: Gram stained HeLa cells infected with *E. coli* strains, exhibiting different adherence patterns (from left to right: diffuse, aggregative, localized)

Concerning the soluble enzymatic virulence factors pattern, the tested strains proved to be positive for mucinase, siderophore-like factors production (esculinase) and lysin-decarboxilase, but negative for the other tested soluble factors.

4. CONCLUSIONS

Our study revealed that the low percentage of *Escherichia coli* resistant strains isolated from sea waters exhibited multiple drug resistance to beta-lactams, tetracyclines, sulphametoxazole and quinolones, correlated with a high tolerance to heavy metals, pleading for an unspecific resistance mechanism, probably mediated by 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 facotrs, such as mucinases and iron-chealting agents. The results of the present study have shown that the 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.

5. REFERENCES

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