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Comparative study of resistance and virulence markers in *Escherichia coli* strains isolated from hospital surfaces, clinical specimens and drinking/ marine waters

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ABSTRACT

The aim of this study was the phenotypic analysis of the antibiotic resistance and virulence markers in enterobacterial strains isolated from hospital surfaces, clinical and water sources. In this purpose, 80 enterobacterial strains were investigated for their susceptibility patterns, for the ability to colonize the cellular (HeLa) and inert substrate and for the production of soluble, enzymatic factors. The enterobacterial strains isolated from different sources exhibited different resistance and virulence patterns. The *E. coli* strains isolated from the hospital environment (both surfaces and clinical sources) exhibited high levels resistance rates to beta-lactams, including 3rd generation cephalosporins, aminoglycosides, fluoroquinolones, tetracyclines, sulphametoxazole and nalidixic acid, as compared to the aquatic strains with much lower resistance rates, to penicillins, tetracyclines and sulphametoxazole. The majority of the tested strains exhibited ability to colonize the inert and cellular substrate. The hospital strains exhibited the ability to produce a series of soluble enzymes implicated in enteric and extra-intestinal pathogenesis (especially pore forming enzymes, proteases and mucinases). The soluble virulence factors expression in the aquatic strains was much poorer. Our results are demonstrating that *E. coli* strains isolated from the hospital environment express phenotypic virulence and resistance markers distinct from those observed in the aquatic strains, demonstrating the adaptation of specific phenotypes to specific ecologic niches. However, the aquatic strains could contribute to the increase of resistance and virulence genes reservoirs with potential implication for the human health in the hospital environment, as demonstrated by the presence of resistance markers, especially in the sea water strains and by the high ability to colonize the abiotic and biotic surfaces.

Keywords: *Escherichia coli, virulence markers, antibiotic resistance markers, hospital surfaces, enterobacterial clinical strains, drinking water, marine water, faecal pollution*

1. Introduction

Enterobacterial strains and especially *Escherichia coli*, normally found in the microbiota of the gastrointestinal tract, are the most frequently etiological agents involved in a large diversity of

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intestinal and extra-intestinal infectious pathology [1-8]. The extensive use of the antimicrobial substances led to the emergence of multiresistant strains, increasing the number of nosocomial infections and complicating their clinical picture [9]. It is also known that the resistance genes occurred in the microbial strains isolated from the hospital environment are originating in the external medium, evolving as non-specific defense mechanisms against the toxic compounds existing in the environment, such as plant metabolites and soil microbiota [10-16]. 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 [17]. *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. 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 species could exhibit powerful virulence factors and can cause severe illness with a large spectrum of symptoms [18]. The aim of the present study was to investigate the antibioresistance and virulence profiles in *E. coli* strains isolated from drinking/sea waters, hospital surfaces and clinical sources.

2. Experimental section

2.1. Bacterial strains. The environmental *E. coli* strains were isolated in Constanta from drinking and sea water. The isolation and identification of these strains are 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. Oxidase and indole production test were performed additionally for the identification of *E. coli* strains. The hospital *E. coli* strains were isolated from urine cultures and respectively from the hospital surfaces on lactose-containing media and identified by conventional biochemical tests (TSI –triple sugar iron, MIU-mobility indole urease, MILF –mobility indole lysin-decarboxylase phenylalanin desaminase, Simmons Citrate), API 20 E and VITEK II automatic system.

2.2. *In vitro* antibiotic susceptibility testing. Bacterial suspensions of 0.5 McFarland density were obtained from 18 h bacterial cultures developed on solid media. The antimicrobial activity was tested on classical Mueller- Hinton agar using standard disk diffusion method and the following antibiotic disks (Oxoid): ampicillin (AM-10μg), ceftazidime (CAZ-10μg), cefuroxime (CXM-10μg),

cefotaxime (CTX-10μg), amoxicillin/clavulanic acid (AMC-10μg), tetracyclines (T-10μg), doxicycline (D-10μg), ticarcillin (TIC-10μg), amikacin (AN-10μg), sulphamethoxazole (STX-10μg), ciprofloxacin (CIP-10μg), pefloxacin (PEF-10μg) and nalidixic acid (NA-10μg) [19-23].

The phenotypic screening of beta-lactamase production and their type was performed by nitrocephine chromogenic test and double disk diffusion test (DDST): the synergism between the AMC and 3rd generation cephalosporins indicated the production of an extended spectrum beta-lactamase (ESBL), while the antagonism between imipenem and 3rd generation cephalosporins the presence of an inducible cephalosporinase [24, 25].

2.3. *In vitro* assessment of the adherence capacity to the cellular substrate. Adherence capacity was qualitatively assayed by Cravioto's adapted method. HeLa cells were routinely grown in Eagle's minimal essential medium (Eagle MEM) supplemented with 10% heat-inactivated (30 min at 56°C) foetal bovine serum (Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), and 0.5 ml of gentamycin (50 μg/ml) (Gibco BRL) and incubated in a 5% CO_2 humidified atmosphere, at 37°C for 24 hrs. HeLa cells monolayers grown in 6 multi-well plastic plates were used at 80% confluence. Bacterial strains from an overnight culture on 2% nutrient agar were diluted at 10^7 CFU/ml in Eagle MEM without antibiotics. The HeLa cell monolayers were washed 3 times with Phosphate Buffered Saline (PBS) and 2 ml from the bacterial suspension were inoculated in each well. The inoculated plates were incubated for 3 hrs at 37ºC. After incubation, the monolayers were washed 3 times with PBS, briefly fixed in cold ethanol (3 minutes), stained with Giemsa stain solution (1:20) (Merck, Darmstadt, Germany) and incubated for 30 min. The plates were washed, dried at room temperature overnight, examined microscopically (magnification, ×2500) with the immersion objective (IO) and photographed with a Contax camera adapted for microscope Zeiss [6, 18].

2.4. The adherence to abiotic surface (slime test). The strains were cultivated in tubes with nutrient broth and incubated at 37°C for 24 hours; the culture tubes were further emptied and stained with safranin alcoholic solution 1 % for 30 min, washed three times with distilled water and left at room temperature for 24 hours. The intensity of the red ring on the tube glass wall was noted with +, ++, +++, ++++ [6, 18].

2.5. Pore forming-toxins. *CAMP-like factor*: the tested strains were streaked at 8 mm distance from the beta-haemolysis producing *Staphylococcus aureus* (ATTC25923) strain on 5% sheep blood agar plates and incubated aerobically at 37 ^{0}C for 24 hours. 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 [6, 26]. *Plate haemolysis*: the strains were streaked on blood agar plates containing 5% (vol/vol) sheep blood in order to obtain isolated colonies. After incubation 24 hours at 37°C, the clear areas (total lysis of red blood cells) around the colonies were registered as positive reactions [6, 26]. *Kanagawa phenomenon*: was tested on Wagatsuma's agar containing 5% rabbit blood. The tested strains were streaked in spots and incubated at 37°C for 24 hours. The areas of beta-haemolysis around the spots indicated positive reactions [6, 26]. *Lipase production*: cultures 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 zone around the spot was registered as positive reaction [6, 26]. *Lecithinase production*: cultures were spotted into 2,5% yolk agar and incubated at 37°C until 7 days. An opaque (precipitation) zone around the spot indicated the lecithinase production [6, 26]. *DN-ase production* was studied on DNA medium. The strains were spotted and after incubation at 37°C for 24 hours, a drop of HCl 1N solution was added upon the spotted cultures, a clearing zone around the culture being interpreted as positive reaction [6, 26].

Proteases. *Caseinase* activity was determined using 15% soluble casein agar as substrate. The strains were spotted and after incubation at 37°C for 24 hours, a clearing zone surrounding the growth indicated casein proteolysis [6, 26]. *Gelatinase* activity was determined on 3% gelatine agar. The strains were spotted and after incubation at 37°C for 24 hours, a precipitation zone around the culture spots indicated gelatin proteolysis [6, 26].

Glucidases. *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 hours at 35°C; 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 [6, 26]. *Amylase* production was tested on 10% starch supplemented agar medium. The strains were stubbed and incubated at 37°C for 24 h, starch hydrolysis being registered by the presence of a clear area around the culture spot [6, 26]. *Aesculin hydrolysis* was tested on iron salts and 1% aesculin medium. After 24 hours incubation at 37°C, the positive strains (aesculin hydrolysis) were recognized by the black color (due to aesculetol-iron salts complexes forming) of the medium [6, 26].

Decarboxilases. LDC–ases are active (efficient) in anaerobiosis, at acid pH and in the presence of phosphate pyridoxal coenzyme, catalyzing the ornitine and lysin (dyamino-monocarboxilic aminoacids) decarboxilation with the production of the specific dyamines. The glucose fermentation by bacteria with fermentative metabolism is inducing the culture medium acidity and the medium color turns in to yellow. If the bacteria do not express lysin decarboxilase 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 [6].

3. Results section

During the present study 80 *E. coli* strains isolated from hospital surfaces (tiles walls, tables, decks beds, blankets, sheets) (20 strains), clinical sources (urine cultures) and drinking/sea waters (20 strains each of them) were analyzed. The *E. coli* strains isolated from hospital surfaces were constitutively resistant to penicillins (98%) and exhibited high resistance rates to 2nd and 3rd generation cephalosporins (98-100%), tetracyclines (46-53%), trimetoprim/sulphametoxazole (38%) and fluoroquinolones (14-24%). The extended spectrum beta-lactamases have been evidenced by double disk diffusion test positive for 36% of the tested strains, while 50% exhibited antagonism aspects between imipenem and 3rd generation cephalosporins, probably due to an inducible AmpC enzyme. The strains those isolated from clinical sources exhibited high resistance rates to aminopenicillins (>90%), 3rd generation cephalosporins (70%), demonstrating a high incidence of ESBL producing *E. coli* strains in the hospital environment), aminoglycosides (70%) and fluoroquinolones (60%). The rate of multiple resistance to aminopenicillins, aminoglicosydes and fluoroquinolones was also high (20%) in the respective strains. The enterobacterial strains isolated from water sources exhibited, as expected, lower resistance rates, as compared with the hospital strains, but however they proved to be resitant to ampicillin, ticarcillin, tetracyclines and sulphametoxazole (~30%) and were susceptible to all other tested antibiotics. 7% of the drinking and 14 % of the marine strains proved to be positive for the presence of beta-lactamases production when tested by nitrocephine rapid test. The microbial adherence to the cellular substrate represents a

sine qua non condition for the colonization and infection process. 35% of the *E. coli* strains isolated from urine cultures exhibited high adherence indexes (80-100%) with all the described adherence patterns (aggregative, localized and diffuse adherence). 45% of the *E. coli* strains isolated from hospital surfaces exhibited adherence ability to the cellular substrate, represented by HeLa cells, out of which 24% with an aggregative and 21% with a diffuse pattern. The presence of the capacity to colonize the cellular substrate in the *E. coli* strains isolated from surface in the hospital environment reflects the ability of these strains to initiate an infectious process in the conditions of colonization of a sensitive host, particularly when the cutaneous -mucous barrier is affected [29-30]. 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) demonstrating the potential of these strains to colonize the animal and human tissues and to initiate an infectious process. The marine water strains exhibited a very low ability of colonizing the cellular substrate. All tested strains exhibited the ability to colonize the inert substratum, feature that explains their ability to persist on the hospital surfaces, medical devices or external medium (inlcuding pipes, water reservoirs, tanks, swimming pools). After the initial colonization and the reach of a certain threshold the bacterial activity is switched on the secretion of soluble virulence factors implicated in infectious process progression and the subsequent invasion of the host organism [29]. The aquatic *E. coli* strains, irrespective to the water sources scarcely expressed soluble enzymatic virulence factors, i.e. the drinking water strains produced lipase and sea water strains produced amylase, which could be implicated either in survival of strains in the external environment, or in the colonization. Out of the total number of *E. coli* strains isolated from the hospital surfaces, 82% produced α-haemolysins, 60% mucinase, 39% caseinase, 8% β-haemolysins and lipase, 7% gelatinase and 4% lecithinase. Over 80% of the *E. coli* strains isolated from the urine cultures expressed DN-ases, β-haemolysins, lecitihinase, caseinase and gelatinase. Ross (1959) demonstrated mucinase activity in different pathovars of *E. coli* and *Shigella* strains of various serotypes but not in normal-flora strains of *E. coli* [31, 32, 33]. Similar activity has been demonstrated in *V. cholerae* [34]. Proteases are extra-cellular enzymes with low substrate specificity are implicated in the destruction of host tissues [31-34]. The mucinase enzyme of this organism has been shown to be active against mucin, but also against lactoferrin and fibronectin and this ability may contribute to pathogenesis. The mucin-degrading enzymes might thus be involved in the breakdown of mucous barriers, thereby allowing bacteria to approach the intestinal membrane, inactivating an unspecific host defence mechanism such as lactoferrin and obtaining aminoacids for its growth. Haemolysins, as well as lecithinases and lipases are implicated either in the bacterial pathogenesis by acting as pore-forming enzymes or in the survival of bacterial strains in the external environment [36-38].

4. Conclusions

The enterobacterial strains isolated from different sources exhibited different resistance and virulence patterns. The *E. coli* strains isolated from the hospital environment (both surfaces and clinical sources) exhibited high levels resistance rates to beta-lactams, including 3rd generation cephalosporins, aminoglycosides, fluoroquinolones, tetracyclines, sulphametoxazole and nalidixic acid, as compared to the aquatic strains with much lower resistance rates, to penicillins, tetracyclines and sulphametoxazole. The majority of the tested strains exhibited ability to colonize the inert and cellular substrata. The hospital strains exhibited the ability to produce a series of soluble enzymes

implicated in enteric and extra-intestinal pathogenesis (especially pore forming enzymes, proteases and mucinases). The soluble virulence factors expression in the aquatic strains was much more diminished. Our results are demonstrating that *E. coli* strains isolated from the hospital environment harbor phenotypic virulence and resistance markers distinct from those observed in the aquatic strains, demonstrating the adaptation of specific phenotypes to particular specific ecologic niches, this adaptability making from the aquatic *E. coli* strains a risk factor for the human health. However, the aquatic strains could contribute to the increase and maintainance of resistance and virulence genes reservoirs with potential implication for the human health in the hospital environment, as demonstrated by the presence of resistance markers, especially in the sea water strains and by the high positivity levels of adherence to abiotic and biotic surfaces.

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