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Virulence markers of *Escherichia coli* strains isolated from hospital and poultry wastewater

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ABSTRACT

The aim of the study was to investigate the presence of several virulence genes and factors associated with virulence in *E. coli* strains isolated from different categories of wastewater. The majority of the aquatic strains isolated from wastewater exhibited high capacity of adherence to the cellular substratum (72.41%) demonstrating the potential to colonize the eukaryotic host cells and to initiate an infectious process, augmented by several extracellular enzymes associated with bacterial pathogenesis and their surveillance in the extreme conditions of the aquatic ecosystem, including: amylase (58%), caseinase (44.82%) and lipase (34.48%). The molecular screening of virulence markers revealed that 44.82% of *E. coli* isolated simultaneously harbor *pldA* and *helD* genes. No specific genes (*aggR, EAggEC, EAST/1, VT1, VT2*) for any of the known *E. coli* pathotypes has been detected in tested strains.

Keywords: *virulence markers, environmental bacteria, virulence genes, wastewater.*

1. INTRODUCTION

E. coli is a major component of the normal intestinal microbiota of humans and other mammals. While most strains of *E. coli* are nonpathogenic, highly pathogenic strains can cause severe illness in humans with a large spectrum of symptoms [1]. Currently, the enteric *E. coli* pathotypes are divided into six groups based on serological and virulence characteristics: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohaemorrhagic (EHEC), enteroaggregative (EaggEC), and diffuse adherent (DAEC) [2]. One enterohaemorrhagic serotype, i.e. *E. coli* O157:H7, has been implicated in many foodborne [3] and a few waterborne outbreaks [4] in Europe, Japan and US.

The new genetic data on pathogenic *E. coli* indicates that most of the additional genes (10-20% of the genomic information) found in pathogenic *E. coli* encode various virulence factors which directly determine their virulence and the respective pathotype [1].The pathogenicity of *E. coli* strains is a complex multi-factorial mechanism involving specific virulence factors which include adhesins, invasins, toxins and capsule [5]. The virulence mechanisms that characterize *Escherichia coli* are genetically coded for by chromosomal, plasmid, and bacteriophage DNAs and include heat-labile (LTI, LTIIa, and LTIIb) and heat-stable (STI and STII) toxins, verotoxin types 1, 2, and 2e (VT1, VT2, and VT2e, respectively), cytotoxic necrotizing factors (CNF1 and CNF2), attaching and effacing mechanisms (eaeA), enteroaggregative mechanisms (Eagg), and enteroinvasive mechanisms (Einv) [6]. The presence of *E. coli* in the environment, in particular in wastewater and then in receiving surface water, is a strong indication of recent faecal contamination and is therefore a good

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indicator of the possible presence of enteric pathogens of human health concern [7]. When this surface waters are used as sources for drinking water production and the water is not treated or inadequately treated, *E. coli* may end up in drinking water [8] or human food chain. The persistence of *E. coli* in biofilms within water distribution system also poses a potential threat to human health [9].

The aim of the study was to investigate the presence of several virulence genes and factors associated with virulence in *E. coli* strains isolated from different categories of wastewater.

2. EXPERIMENTAL SECTION

2.1. Isolation and identification of bacterial strains. In this study, there were analyzed 29 environmental *E. coli* strains isolated from hospital untreated wastewater (11) and poultry wastewater (18). Water samples (500 ml) were collected according to ISO 19458/2006 using sterile glass bottles, stored in cold bags at 4^0C and transported to the laboratory for analysis within 6 h of collection. The isolation and identification of *E. coli* strains is based on standardized membrane filter method, using cellulose nitrate filters of 0.45 μm pore size, Millipore, USA, according to SR ISO 9308-1/2004 [10]. The membrane is applied on Lactose TTC (triphenyltetrazoliumchloride) Agar with Tergitol 7 (sodium heptadecylsulfate) medium (Merck) poured on 47 mm diameter Petri plates. After 48 hours incubation at 37°C, *Escherichia coli* will develop yellow colonies on the membrane. Preliminary identification of *Escherichia coli* strains obtained in pure culture was based on oxidase reaction and indole production. The bacterial isolates were further identified using biochemical tests: API 20 E (bioMérieux). The identified strains were preserved at -70^0C in LB broth supplemented with 15% (v/v) glycerol and/or semisolid nutrient medium at 4^0C.

2.2. Detection of virulence factors. The *adherence to the biotic substrate* (HeLa cells) was investigated by the Cravioto adapted method [11]. In this purpose, 1 ml bacterial suspension prepared from a broth culture of 24 h was inoculated on a (80%) confluent cellular layer of HeLa-2 cells. After 2 hours incubation at 37^0C, the bacterial suspension was discarded and the cell culture washed and stained by Giemsa method. The adherence was microscopically examined from qualitative (different adherence patterns) and quantitative (adherence index) points of view. The bacterial *ability to colonize the inert substratum* (*slime* test) was quantified by biofilm microtiter method [12]. Microbial suspension with a density of 0.5 McFarland were placed in nutrient broth in multi-well plate and incubated at 37^0C for 24 h and thereafter the cultures were removed, and the plate was washed there times with sterile water and fixed with cold methanol for 5 minutes. Bacterial cells were stained with 1% crystal violet for 15 minutes, then washed with sterile water and resuspended in 33% acetic acid solution. The biomass was assessed spectrophotometrically, by measuring the absorbance at 490 nm, for the obtained coloured suspension.

2.3. Soluble enzymatic factors implicated in bacterial virulence. To detect *haemolysin*, strains were seeded on blood agar containing 5% (vol/vol) sheep blood. 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 [13, 14]. 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 [13, 14]. 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 [13, 14]. 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 [13, 14]. 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 [13, 14]. *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].. The *amylase* production was tested on 10% starch supplemented agar medium. The strains were stubbed and incubated at 37^0C for 24 h, starch hydrolysis was registered by the presence of a clear area around the culture spot [13, 14]. *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 colour (due to aesculetol-iron salts complexes forming) of the medium [13, 14].

2.4. DNA isolation and PCR for virulence genes detection. The genes encoding the expression of some virulence factors to *E. coli* strains were determined using primers previously described (Table 1). Single (*aggR, EAggEC, EAST/1, VT1, VT2*) and multiplex PCR (*pldA* and *heldD*) were carried out using PCR conditions listed in Table 2. Total DNA extraction was performed by a centrifugation step of the cell suspension in BHI (Brain Heart Infusion) broth at 13,000 x g for 10 min. Supernatants were discarded, and the pellets were washed two times with phosphate-buffered saline (120 mM NaH_2PO_4 [pH 8.0], 0.85% NaCl) before extraction of total DNA by boiling technique that includes a heating step at 100^0C for 10 min. DNA was stored at -20°C until use.

Table 1: PCR primers used for detection of virulence genes in *E. coli* strains

Virulence genes	Primer sequence (5' – 3')	Product size (pb)	Reference
aggR	AGACGCCTAAAGGATGCCC GAGTTATCAAGCAACAGCAATGC	430	[15]
EaggEC	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	630	[16]
EAST/1	CCATCAACACAGTATATCCGA GGTCGCGAGTGACGGCTTTGT	111	[17]
VT1	GAAGAGTCCGTGGGATTACG AGCGATGCAGCAGCTATTAATAA	130	[18]
VT2	AAGAAGATGTTTATGGCGGT CACGAATCAGGTTATGCCTC	346	[18]
pldA	CAGGGCTGGTTGTTGCCGGT ACGCCACAGCGGAAATGCCA	284	[19]
helD	GGTTGCTGGCGCGTGGTGAA GCGTGAGGCAAGACGACGCT	370	[19]

Table 2: PCR conditions used for detection of virulence genes in *E. coli* strains

Genes	*pldA*	*helD*	*aggR*	*EaggEC*	*EAST/1*	*VT1*	*VT2*
Initial denaturation	95^0C for 5 min	95^0C for 5 min	94^0C for 5 min	94^0C for 5 min	94^0C for 5 min	94^0C for 5 min	94^0C for 5 min
Denaturation in each cycle	95^0C for 1 min	95^0C for 1 min	94^0C for 30 s	94^0C for 40 s	95^0C for 30 s	94^0C for 2 min	94^0C for 2 min
Annealing	60^0C for 40 s	60^0C for 40 s	57^0C for 1 min	53^0C for 1 min	55^0C for 2 min	55^0C for 1 min	55^0C for 1 min
Extension	72^0C for 1 min	72^0C for 1 min	72^0C for 40 s	72^0C for 4 min	72^0C for 2 min	72^0C for 1 min	72^0C for 1 min
Final extension	72^0C for 5 min	72^0C for 5 min	72^0C for 5 min	72^0C for 5 min	72^0C for 5 min	72^0C for 5 min	72^0C for 5 min
Number of cycles	29	29	30	30	30	30	30

All the PCR assays were carried out in a total volume of 25 µl mixture containing the following reagents: Dream Taq DNA Polymerase supplied in 2X DreamTaq Green buffer (ThermoScientific),

400 µM of each dNTP, 4 mM $MgCl_2$, 0.5 µM of each primer, and 20 ng of genomic DNA (i.e. 1 µl of sample). PCR amplification was performed with Palm Cycler (Corbett Scientific). The PCR products were analysed by electrophoresis in 1.5% agarose gel in TBE1x (pH= 8.3), stained with ethidium bromide and detected by UV transillumination.

3. RESULTS SECTION

3.1. Adherence to the cellular substratum (HeLa cells). The ability to colonize the cellular substratum was 81.81% in *E. coli* strains isolated from hospital untreated wastewater and 61.61% in strains isolated from poultry wastewater. Aggregative and localized adherence patterns were predominant in hospital wastewater (average adherence index of 52.11%) and localized adherence patterns was predominant in strains isolated from poultry wastewater (average adherence index of 22.41%).

3.2. Adherence to the inert substratum. Aquatic *E. coli* strains showed high colonization ability of the inert substratum as demonstrated by the high positivity rate of *slime* test (100%), with average absorbance values ranging from 0.187 for hospital wastewater strains to 0.114 for poultry wastewater strains.

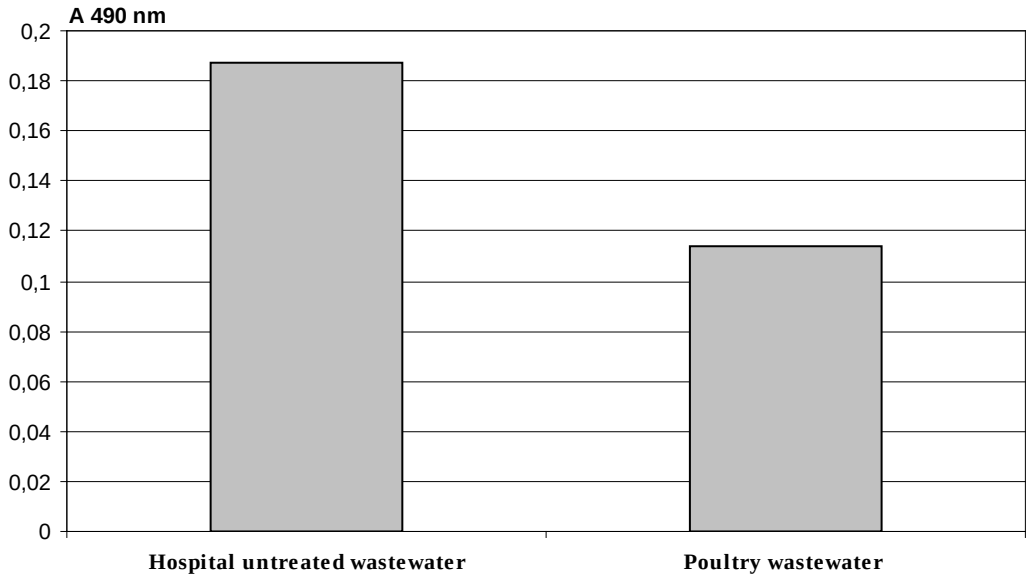

Figure 1: Adherence to the inert substratum (Biofilm microtiter method)

3.3. Soluble enzymatic virulence factors. The tested *E. coli* strains expressed majority of soluble enzymes associated with bacterial pathogenesis and their surveillance in aquatic environment, i.e., amylase (63.63%), caseinase (63.63%), gelatinase and lecithinase (27.27%), in strains isolated from hospital wastewater and amylase (55.55%), caseinase (33.33%) and lipase (44.44%), in strains isolated from poultry wastewater. Esculinase and DN-ase were absent.

3.4. Detection of virulence genes (*aggR, EaggEC, EAST1, VT1, VT2, pldA* and *helD*). The *pldA* and *helD* gene was detected in 54.55% of the strains isolated from hospital wastewater and in 38.88% of the strains isolated from poultry effluent. Specific genes of the known *E. coli* pathotypes (*aggR, EAggEC, EAST/1, VT1, VT2*) have not been detected in tested strains. While the possession of any one of virulence factors does not mean that a microorganism can cause disease, it is generally believed that their known association with pathogenesis is one requirement for the development of infection. The 100% of *E. coli* strains isolated from wastewater colonized the inert substratum, ability that explains their surveillance in the extreme conditions of the aquatic ecosystem, adherence

on abiotic surfaces representing an ecological advantage for bacteria to ensure nutrients in addition to protect the bacterial cells from desiccation effects.

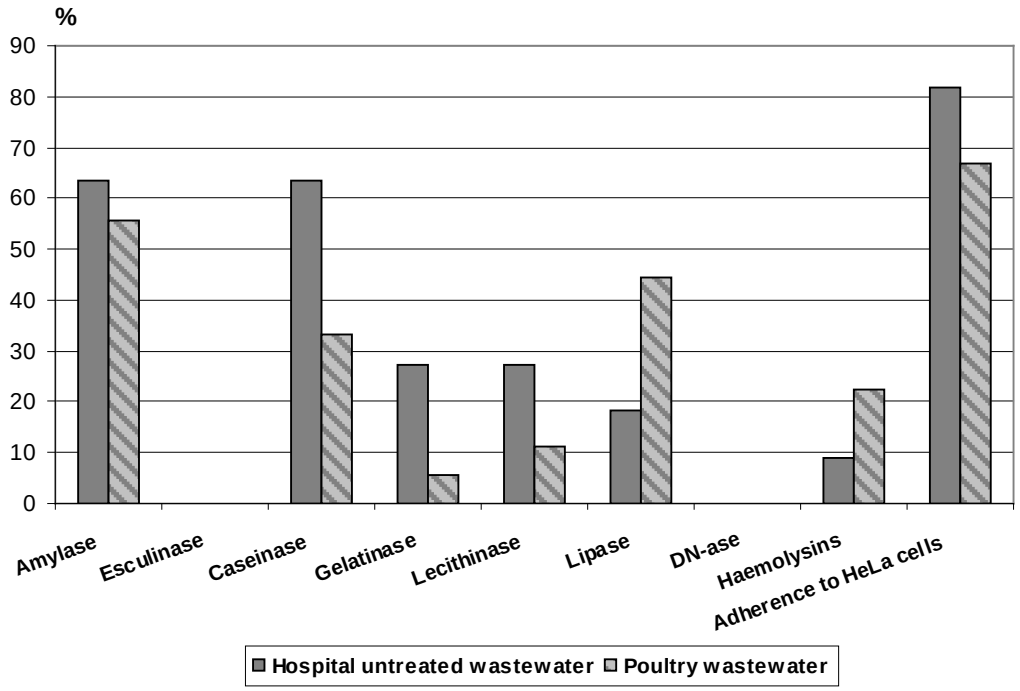

Figure 2: Expression level of different virulence factors in environmental *E. coli* strains

Table 3: Detection of virulence genes in *E. coli* strains

Source of strains	No. of tested strains	No. of positive strains for genes encoding different virulence factors						
		pldA	*helD*	*aggR*	*EaggEC*	*EAST/1*	*VT1*	*VT2*
Hospital untreated wastewater	11	6	6	0	0	0	0	0
Poultry wastewater	28	7	7	0	0	0	0	0
Total	29	13	13	0	0	0	0	0

Furthermore, production of *slime* might be a virulence factor in the case of infection of a host organism by blocking the phagocytosis [12]. The presence of adherence capacity to HeLa cells in environmental *E. coli* strains (72.41%) demonstrated the potential of the respective strains to initiate an infectious process when transferred from the external medium to the animal/human host. A key step in the pathogenesis of nearly all bacterial enteric pathogens is the colonization of the gastrointestinal mucosa, which is mediated by specific adherence factors [20].

The predominant adherence phenotypes in tested *E. coli* strains were those described for two of the known enteropathogenic *E. coli* pathotypes, i.e. EaggEC and EPEC. EAggEC strains are characterized by their unique patterns of adherence to HeLa-2 cells [21], the bacteria appearing as ‘‘stacked brick’’ clumps [22]. This characteristic aggregative adherence pattern is associated with the presence of large plasmids [20], which are closely linked to virulence mechanism, on these plasmids being identified genes encoding bundle-forming fimbriae involved in adherence [23] and a heat-stable enterotoxin (EAST1) [24, 25].

Amylase proved to be the most constant enzymatic virulence factor in *E. coli* strains isolated from wastewater, suggesting the implication of this enzyme in the persistence and surviving of microorganisms in the external environment through the colonization of surfaces (aquatic plants,

zooplankton) and providing nutritional resources in environments with limited nutritional resources such as aquatic environment.

E. coli strains exibited caseinase (44.82%) and gelatinase (13.79%), extracellular enzymes with low specificity that hydrolyze proteins to peptide, factors also involved in the survival and maintenance of pool microorganisms in the environment. Furthermore, gelatinases is a matrix metalloprotease that plays an important role in tissue destruction and subsequent invasion of the host [12].

E. coli strains isolated from wastewater produced haemolysins (17.24%), extracellular enzymes that act in the pathogenesis of bacterial species by production of tissue damage (pore-forming toxins). This action adds to the effect of lecithinases (17.24%) and lipases (34.48%) in disruption of host cell membrane by affecting membrane lipids, suggesting the implication of these enzymes in host invasion.

These phenotypic findings are correlated with the *pldA* gene detection, encoding an outer membrane phospholipase A protein (figure 3). The exact function of the phospholipase A protein (PldA) has not been fully elucidated, although sequence analysis of several enterobacterial *pldA* genes demonstrated that the protein is highly conserved among Enterobacteriaceae, including pathogenic species, and may be essential for growth in natural environment [26], providing a favourable source of nutrients for bacterial growth and a suitable environment for replication and survival [27] and for membrane disruption processes that often occur during host cell invasion [28]. Phospholipases have often been implicated as virulence factors in other bacterial pathogens, i.e. in *Rickettsia prowazekii*, an obligate intracellular parasite, phospholipase A being thought to be involved in bacterial escape from the host cell phagosome [29]. Furthermore, some studies correlate the ability of bacteria to lyse erythrocytes with phospholipase activity [30]. Grant et al. [31] demonstrated that the *pldA Campylobacter coli* mutants have a reduced hemolytic activity compared to the wild-type strain, suggesting the role for the phospholipase A in the lysis of erythrocytes. Furthermore, in *E. coli*, the PldA protein was found to be implicated in generating small holes in the outer membrane to allow the escape of bacteriocins, comparing with PldA-deficient mutants [32].

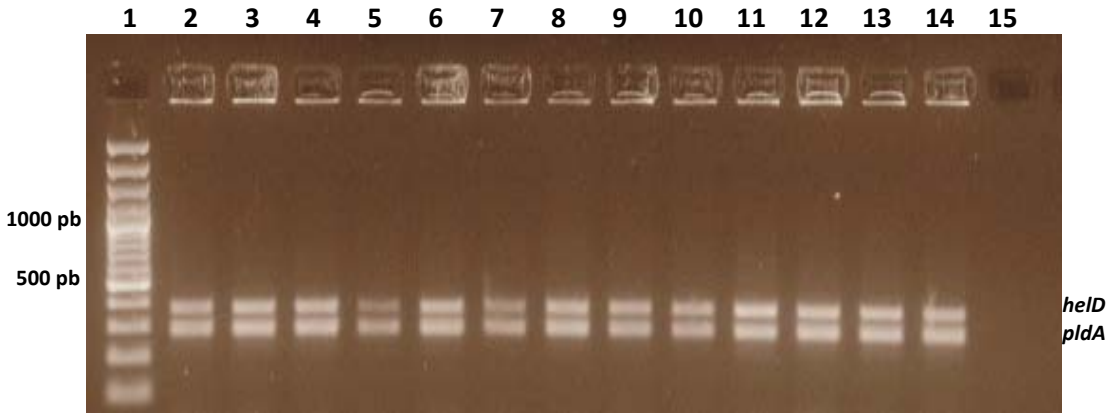

Figure 3: Gel electrophoresis of the PCR products of *helD* (370 pb) and *pldA* (284 pb) genes amplification for different *E. coli* strains isolated from wastewater

1: Molecular size marker (Fermentas); 2: *E. coli* N61 HW; 3: *E. coli* N73 HW; 4: *E. coli* N62 HW; 5: *E. coli* M112 PW; 6: *E. coli* N79 HW; 7: *E. coli* K1 PW; 8: *E. coli* K7 PW; 9: *E. coli* J33 PW; 10: *E. coli* K8 PW; 11: *E. coli* L13 PW; 12: *E. coli* N72 HW; 13: *E. coli* N65 HW; 14: *E. coli* N70 HW; 15: Negativ control

HW=hospital untreated wastewater; PW= poultry wastewater

The *helD* gene was amplified in 44.82% of the *E. coli* strains. This gene is encoding the helicase IV, whose role is not fully elucidated [33]. In *Escherichia coli* strains there have been purified and

characterized at least 10 distinct helicases implicated in nucleic acid metabolism [34]. A *helD* null mutant strain showed about 10-fold decreased frequencies of recombination [33]. It has been noted that helicase IV shares several physical and biochemical properties with helicase II and DNA replications proteins. Helicase II, has been shown to have roles in both methyl-directed mismatch repair and excision repair pathways [35]. However, the presence of this gene was not reflected in the phenotypic ability of the tested strains to produce DN-ases. This result could be explained by the fact that DN-ases are specifically recognize and cut DNA at the level of certain sequences [36], resulting in DNA fragments of different sizes. In the phenotypic assay, the positive results implicate the total depolymerisation of DNA, with the accumulation of purinic and pirimidinic monomers which, due to the free phosphate groups are inducing the changes of the medium color [13].

Few studies provide information on the occurrence of pathogenic *E. coli* harbouring specific genes for known pathotypes of *E. coli* in surface water [37, 38, 39]. In this study, specific genes of enteroaggregative *E. coli* pathotype (*aggR, EAggEC, EAST/1*) and enterohaemorrhagic *E. coli* pathotype (*VT1, VT2*) have not been detected in tested strains. Similar, Panus et al. [14], investigated some virulence markers in *E. coli* strains isolated from sea water and all tested strains were negative for the transcriptional activator for EAggEC aggregative adherence fimbriae I expression (*aggR*), and *E. coli* heat-stable enterotoxin *1 (EAST/1).*

4. CONCLUSIONS

The results of the present study have shown that *E. coli* strains isolated from wastewater have harboured virulence markers (ability to colonize the cellular and substrata, pore-forming enzymes and proteases), which constitutes a basis for the selection and spread of virulence determinants and might contribute to the emergence of pathogenic *E. coli* strains, with an increased risk for the human health.

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