ORIGINAL ARTICLE

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Extended spectrum beta-lactamases in *Escherichia coli* from municipal wastewater

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Abstract

Introduction and objective. Over the past decades, awareness of the environmental load of resistant organisms has increased. The presented paper focuses on antibiotic resistance and detection of resistance genes in environmental *E. coli* and on the evaluation of biofilm formation in ESBLs (extended spectrum beta-lactamase) producing *E. coli* isolated from an urban wastewater treatment plant.

Materials and method. Wastewater samples and artificially added polystyrene pellets were used as the source for *E. coli* isolation. Minimal inhibitory concentrations of 19 antibiotics were determined according to CLSI (2013). Biofilm formation was investigated by crystal violet or resazurin methods. CTX-M, carbapenemases, *qnrS*, mobile elements and virulence factors were determined by PCR. Clonal relatedness of strains was detected by principal component analysis by a Maldi biotyper.

Results. ESBL phenotype was detected in 26% of environmental strains. CTX-M, CMY-2 and *qnrS* genes of antibiotic resistance were detected. IMP gene together with integron 1 in one ertapenem resistant *E. coli* was also recorded. There was no evident correlation between antibiotic resistance, virulence and biofilm production.

Conclusions. The results showed that the wastewater is a source of ESBLs, carbapenemases and plasmid fluoroquinolone resistance. Strains with biofilm production, antibiotic resistance of CTX-M group, CMY-2, *qnrS* genes and virulence factors present a potential environmental health risk.

Key words

E.coli, ESBL, virulence, biofilm

INTRODUCTION

The excessive use and misuse of antimicrobial agents in human and veterinary medicine, animal farming, industrial settings, and their subsequent release to wastewater and wastewater treatment plants (WWTPs) have contributed to the emergence and dissemination of resistant bacteria into the environment [1]. Many pathogenic bacteria are able to form biofilms, and thus develop resistance to multiple antibiotics and to synthesize cell surface components that help them survive in hostile or suboptimal environments [2]. The association between biofilm formation and virulence factors was reported as variable [3]. The prevalence of Enterobacteriaceae (e.g., *Klebsiella pneumoniae* and *Escherichia coli*), producing extended spectrum betalactamases (ESBLs) and carbapenemases, has rapidly increased during the last decade.

OBJECTIVE

The aim of the study was to investigate antibiotic resistance (ESBLs) and to determine a possible correlation between biofilm ability and virulence or resistance genes in *E. coli* isolated from a municipal waste water treatment plant.

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MATERIAL AND METHODS

Environmental sampling. The study was based on the detection of antibiotic resistance and ESBLs production in *E. coli* isolated from wastewater treated in a municipal WWTP in Slovakia. The plant is located in a village situated close to Košice in eastern Slovakia. Samples of wastewater were taken from the influents and effluents of the WWTP in 2011–2013, at different times of the year, predominantly from April – November. Throughout the study, 104 strains of *E. coli* were investigated. Polystyrene pellets added to wastewater in the municipal wastewater treatment plant were used to isolate biofilm producing *E. coli*.

Cultivation. Each sample of wastewater from the influent or effluent and polystyrene pellets were inoculated and multiplied in Buffered peptone water (Oxoid, Basingstoke, UK) and then were subcultured on Mac Conkey agar (Oxoid) overnight at 37 °C. Identification of bacteria was performed by a Maldi Tof biotyper [4].

Antibiotic sensitivity. Minimal inhibitory concentrations (MIC) were determined according to VET01-S2 (CLSI, 2013) [5]. The antibiotics used in the presented study were as follows: ampicillin (AMP); ampicillin+sulbactam (A+IB); ertapenem (ETP), ceftiofur (CFT); ceftriaxone (CTR); ceftazidime (CAZ); cefquinome (CFQ); gentamicin (GEN); streptomycin (STM); neomycin (NEO); spectinomycin (SPE); nalidixic acid (NAL); enrofloxacin (ENR); ciprofloxacin (CIP); chloramphenicol (CMP); florphenicol (FLO); tetracycline

(TTC); trimethoprim+sulphonamide (COT), colistin (COL), mentioned in the study by Gregova et al., 2012 [6]. Phenotype interpretation of mechanisms of ESBLs were read according to the β-lactams (CTR, CAZ, CAC) MIC levels [6].

Detection of biofilm. For the detection of biofilm formation, crystal violet or resasurin methods with Nunc Maxisorp plates were used. The crystal violet method consists of 24 h *E.coli* incubation at 37°C, washing 3 times with saline, fixing with methanol and staining with 1% crystal violet. The bound dye was released with 33% glacial acetic acid, and optical density (OD) at 570 nm was measured by using an ELISA reader [7].

Resazurin assay detects live biofilm bacteria. After *E. coli* cultivation and washing, 0.02% resazurin solution was added, the samples incubated for 30–240 min at 37 °C and changes of colour from blue resazurin to red resorufin observed [8]. Strong biofilm producers changed colour within 30 min, medium after one hour, and weak producers after more than 1 hour.

PCR. ESBL genes for CTX-M [9], CMY-2 (Cit) [10], gnrS [11], integrase 1 [12] and carbapenemases (KPC, NDM, VIM, IMP, OXA-48) [13] were determined by PCR.

Genes of virulence factors for APEC (avian pathogenic *E. coli*), e.g. *iutA*- receptor for aerobactin, *kpsII*-capsular polysialic acid virulence factor and *cvaC*-colicin V, were investigated according to Johnston and Stell [14], *iss*-increase serum survival according to Foley et al. [15], *tsh*-temperature sensitive haemagglutinin by the method of Dozois et al. [16], *papC*-P fimbriae by the method of Le Bouguénec et al. [17], microcin H47 and *ColE1* according to Gordon and O'Brien [18].

Typisation of strains. *Escherichia coli* isolates were assigned to the pathogen group (B2 and D) and commensal group (A and B1) according to the method by Clermont et al. [19]. Clonal relatedness of *E. coli* was measured by principal component analysis (PCA) using a Maldi biotyper [4].

RESULTS

The resistance to 19 antibiotics and MIC90 levels in 104 isolates *E. coli* detected in samples from municipal WWTP in the period of two years is shown in Figure 1.

The highest incidence of beta-lactams resistance was observed for ampicillin (88%), followed by cephalosporins – veterinary ceftiofur (52%) and cefquinom (14%), ceftriaxone (34%) and ceftazidime only 1.9% of all strains. Three strains were resistant to ertapenem. ESBL phenotype was present in 26% of strains and MIC90 for ESBL indicator antibiotics were for ceftazidime 8 mg/L and for ceftriaxone 128 mg/L.

Enrofloxacin resistance occured in 50% of strains and ciprofloxacin resistance in 49% of strains. MIC 90 of enrofloxacin in 104 strains *E. coli* resistant isolates was very high and reached 32 mg/L and for ciprofloxacin 8 mg/L.

Seventeen strains of *E. coli* were selected for PCR experiments on the basis of antibiotic resistance and biofilm production. These 17 *E. coli* strains, obtained from influents and effluents of municipal waste water treatment plant, were divided into four groups on the basis of biofilm production: strains with strong, moderate and with weak

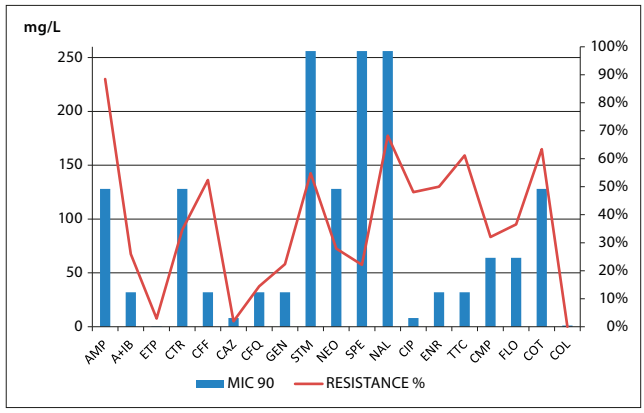

Figure 1. Antibiotic resistance and MIC90 levels in 104 environmental *E. coli*

biofilm production, and strains without biofilm production. Investigation of these strains tested for biofilm formation showed the presence of ESBL CTX-M type in approximately half of the strains. The majority of ESBL producing *E. coli* showed weak biofilm production and belonged to a commensal phylogenetic group. However, one *E. coli* strain, 17 B (isolated from polystyrene pellets), with moderate biofilm activity, contained four resistance genes CTX-M1, CTX-M2, CMY-2 and plasmid quinolone resistance *qnrS* together with integron 1 and transposon 3 and the virulence genes *iutA*, *fimA*, *kps*, *papC*, *ColE1* (Tab. 1).

In one environmental isolate, *E. coli* 7, the presence of metallo-beta-lactamase IMP-type and integron 1, manifested with weak biofilm formation, was identified, and included among the commensals. The other two pathogenic strains, 14 and 15, resistant to ertapenem, showed the presence of CTX-M and integron 1 without, however, biofilm production.

Table 1. Genotyping and biofilm production in selected *E. coli* strains

Strains with weak biofilm production	**Phylogenetic Group**
CTX-M1, Int1,Tn3, *iutA,*	1xA (commensal)
CTX-M1, Int1, *iutA,*	1xA
CTX-M1, Int1, *fimA*, *aer*, *iutA,*	**1xB2**
CTX-M1, Int1, *fimA*, *afa*, *aer*, *iut A,*	1xA
CTX-M1, *iutA,*	**1x D (pathogen)**
Cit, Int1,	1xA
IMP, Int1	1xA
qnrS	1xA
micrH47	1xA
Strains with moderate biofilm production	
CTX-M1, CTX-M2, Cit, *qnrS*, Int1, Tn3, *iutA*, *fimA*, *kps*, *papC*, *ColE1*	**1xND**
fimA, *aer*, *papC*	1xND
Strains with strong biofilm production	
fimA, *aer*	1xND
without genes	1x ND
Strains without biofilm production	
CTX-M1,Cit, Int, *iutA*	**1xB2 (pathogen)**
CTX-M1, Int1, Tn3, *papC*	**1xB2**
CTX-M1, Tn3, *fimA*, *sfa*, *papC*, *micrH47*	**1xB2**
Int1, Tn3, *fimA*	1xA

ND – not detected

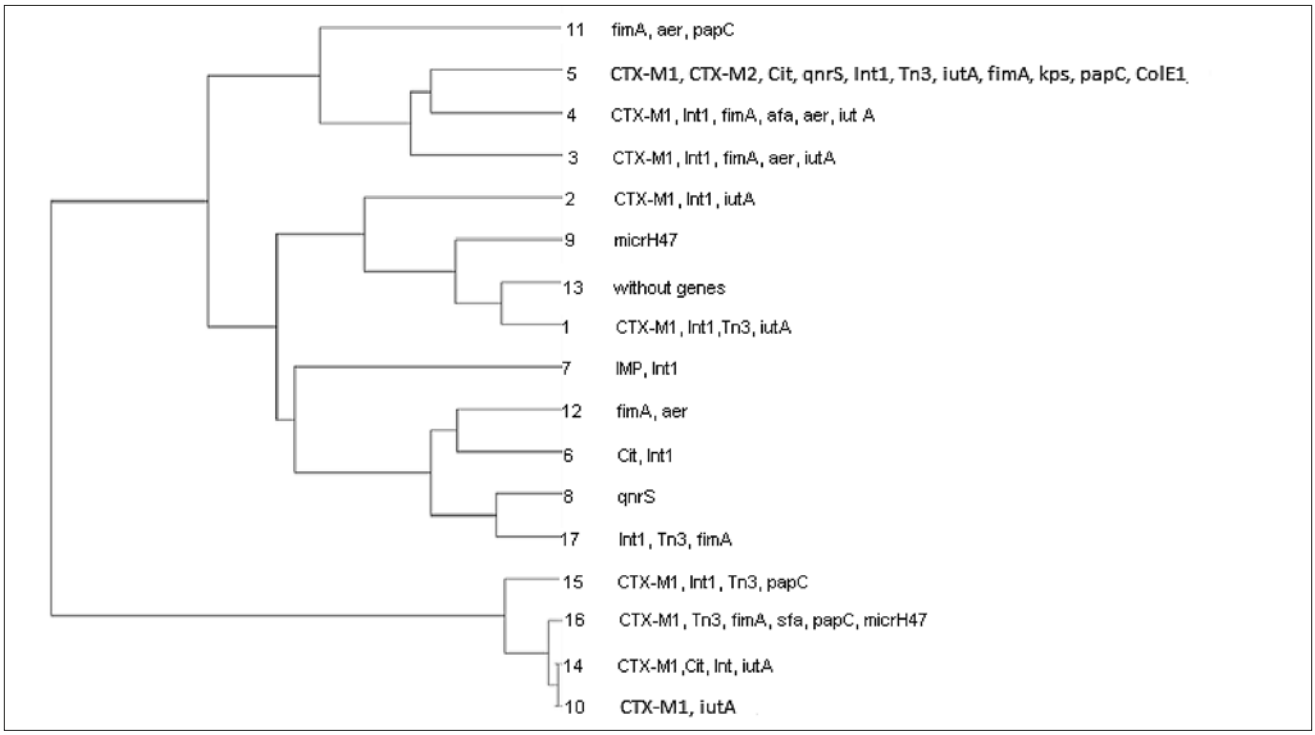

Figure 2. Principal component analysis of 17 environmental *Escherichia coli*

The characterization of 17 ESBL-producing *E. coli* isolates from WWTP is shown in Figure 1. Principal component analysis revealed three clusters of strains. *E.coli* 17 B is No.5 and *E. coli* 34 (strong biofilm) is No. 12 (Fig. 2). The dendrogram presents all investigated strains with their properties and their ability to form biofilm formation (Tab. 1).

DISCUSSION

Over the past decades, awareness of the environmental load of resistant organisms has increased. The results of the presented study indicate that wastewater treatment plants can be a source of antibiotic-resistant and virulent *E. coli*, especially CTX-M, CMY-2 and *qnrS*. Similar results with a high level of ESBL phenotypes in animal *E. coli* isolated from poultry slaughterhouse has been described by Gregova et al. [6]. *E. coli* from rivers and lakes in Switzerland with ESBL genes, produced CTX-M-1, CTX-M-3, 46, CTX-M-14, CTX-M-15, CTX-M-27, CTX-M-55, and CTX-M-79. The CTX-M-27 producers belonged to the multi-resistant pandemic sequence type *E.coli* B2:ST131, that is strongly associated with potentially severe infections in humans and animals [20]. The water in fountains should be also carefully monitored for the presence of microorganisms, because they often serve as drinking bowls for birds. Kmet et al [4] reported a high occurrence of CTX-M in *E. coli* from rooks. The common use of water reservoirs by humans and animals may contribute to the transmission of pathogenic species of microorganisms [21].

Acquired carbapenemases, type IMP, from non-human sources were found in sewage in Germany. In the current study, the IMP gene with integron 1 was found in only one environmental *E. coli* isolate. Other types of carbapenemases, e.g. KPC, VIM, OXA-48, have been found in effluents and rivers in Europe [22]. However, the animal origin of carbapenem resistant strains is uncertain because carbapenems are not registered for animal therapy. In humans, carbapenemase-producing Enterobacteriaceae may be either hospital acquired (mostly *K. pneumoniae*), or community acquired (mostly *E. coli*), either as colonizers or infectious agents [23].

The formation of a biofilm is a universal bacterial survival strategy. There are two groups of data on the correlation of biofilm production and virulence or antibiotic resistance (positive and negative correlation). Naves et al. [3] showed that five virulence-associated genes were more common among strong biofilm producers: *papC*, *papG* alleles, *sfa/focDE, focG, hlyA* and *cnf1*. In the presented study, *papC* allela were found in only two pathogenic strains without production of biofilm, and in one strain with moderate production of biofilm. Mliji et al. [24] demostrated by *Salmonella* plasmid transformation to *E. coli* DH10B that there is an association between resistance to expanded-spectrum cephalosporin, cell surface proteins and biofilm formation.

Type 1 fimbriae (*fimA*) are the most common among *E. coli* and play an important role in the initial attachment to abiotic surfaces during biofilm formation [25]. However, in the current study, the *fimA* gene was found in both the strain with strong biofilm formation and that without biofilm production. By evaluating the correlation between resistance, virulence and biofilm formation, it was found that the relationship is variable. However, the environmental origin of the studied *E.coli* strains subjected to no or low antibiotic selection pressure could result in biofilm properties differing from those of clinical strains under antibiotic therapy.

CONCLUSION

The results obtained show that urban waste water may be a source of ESBLs, carbapenemases and plasmid

fluoroquinolone resistance strains with biofilm production, antibiotic resistance of CTX-M group, CMY-2, *qnrS* genes and virulence factors, and presents a potential environmental health risk. The genes associated with mobile elements (integron 1 and Tn3) facilitate their spread in the population and horizontal transfer to other organisms. Evaluation of the correlation between antibiotic resistance, virulence, and biofilm formation showed that the relationship between them was variable.

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