ANTIBIOTIC RESISTANT BACTERIA IN BIOFILM FROM SEWERAGE

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ABSTRACT

Nowadays emergence of antibiotic resistant bacteria in the environment represents a serious socio-economic problem. Wastewater on the influent of wastewater treatment plant contains high number of antibiotic resistant bacterial strains, as well as many antibiotics in subinhibitory concentrations. These two factors can contribute to dissemination of antimicrobial resistance in water and consequently in whole environment. Some bacteria in water can attach to the surfaces and form biofilm, which gives bacteria advantage in fight against environmental stress and some other lethal factors. High bacterial density and diversity in biofilms from wastewater generate suitable conditions for horizontal gene transfer and for rise of resistant microorganisms. In our study we have focused on biofilms isolated from influent and effluent sewerage of wastewater treatment plant, which treats water from the biggest settlement in Eastern Europe. Bacterial composition was detected by DGGE fingerprinting coupled with the construction of 16S rRNA clone libraries. According to higher concentrations of antibiotics and different antibiotic resistant bacteria in wastewater, we were also interested in percentage of antibiotic resistant coliforms, enterococci and staphylococci in isolated biofilms. Antibiotic resistance was evaluated according to European (EUCAST) as well as to US (CLSI) resistance breakpoints.

Keywords: biofilm, antibiotic resistance, wastewater, DGGE fingerprinting, clone libraries

1. Introduction

At the present time a variety pharmaceuticals are in use for medical purposes as diagnostics and disinfectants. After application, many drugs are excreted non-metabolized by the patients and enter into wastewater. After their use and sometimes as residual quantities, diagnostic agents and disinfectants also reach the wastewater (Kümmerer, 2001). The occurrence of these compounds in subinhibitory concentrations may promote the selection of antibiotic resistance genes and antibiotic resistant bacteria, which shade health risks to humans and animals. On the other hand, many reports have also documented presence of resistant bacteria in the stool of asymptomatic individuals without antibiotic treatment (Bates, 1997). These bacteria get into wastewater and can spread antibiotic resistance on susceptible microorganisms. Some bacteria are able to attach to surface and form biofilm. High bacterial density and diversity are found in biofilms from wastewater systems, especially from activated sludge of sewage treatment plants. Biofilms are also generated in surface water and drinking water distribution systems (Schwartz, 2003). It is also well known that microorganisms attached to a surface in the form of a biofilm exhibit remarkable resistance to all types of antimicrobial challenge, compared with the same microorganisms grown as freely suspended cells (Mara and Horan, 2003).

The aim of this study was to detect and compare microbiota of biofilm from influent and effluent sewerage, using culture-independent and culture-dependent approaches. However enterococci, coliforms and staphylococci were found in many biofilms, we have also targeted on quantification of these species resistant to antibiotics in influent wastewater and biofilm from influent and effluent sewage.

2. Material and methods

2.1. Wastewater treatment plants and sampling

Samples of influent water and biofilm were collected from WWTPs which receive wastewater from urban households, industries, hospitals and other facilities. This plant A currently serves for 125 000 inhabitants and treats up to 33000 m^3/day of municipal wastewater. The treatment process in plant includes mechanical pre-treatment and an activated sludge system which is followed by a secondary clarifier. Sludge is anaerobically stabilized and the generated biogas is processed to energy. Sampling points were situated on the influent and effluent stream. Samples of influent water were taken every 15 minutes by an automatic sampling device and mixed as 24-hours composite samples. Samples of biofilms were taken from the influent and effluent sewerage. The samples were immediately transported in sterile falcon tubes to the laboratory for microbiological analysis. Samples were collected in October 2014.

2.2. Antibiotic resistance detection

Wastewater sample was diluted in physiological saline and appropriate dilutions were applied on antibiotic and antibiotic free selective diagnostic media. Chromocult Coliform agar (Merck, Germany) was applied for detection of coliform bacteria, SlanetzBartley agar (Biolife, Italy) for enterococci and BairdParker agar (Biolife, Italy) for staphylococci. In case of collected biofilm, 1g of the sample was mixed with 10 mL of physiological saline. Serial 10-fold dilutions of samples in physiological saline were prepared and 0.1 mL aliquots were spread on selective diagnostic agar plates. Antibiotic resistant strains were detected after the cultivation on plates containing diagnostic media with different antibiotics. Before bacteriological counting, the plates were incubated for 24h at 37°C for coliforms and staphylococci and for 48h at 42°C for fecal streptococci. Each experiment was run in triplicate and was repeated three times.

2.3. DGGE analysis and clone library construction

Genomic DNA was extracted from 25 mg of influent and effluent sample, using the Power Soil kit (Mo Bio Laboratories, Carlsbad, USA) according the manufacture instructions. The 16S rRNA bacterial DGGE fingerprinting and the correspondent clone library construction was performed according the approach proposed by Pangallo *et al*. (2014). In this case the semi-nested PCR strategy required the primers 27F (5`-AGA GTT TGA TCM TGG CTC AG- 3`), and 685R (5`-TCT ACG CAT TTC ACC GCT AC- 3`) (Lane, 1991) for the first PCR step and the primers 518F (5`-CCA GCA GCC GCG GTA AT-3`) (Muyzer *et al*., 1993), 685R-GC (5`-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GTC TAC GCA TTT CAC CGC TAC-3`) (Lane, 1991) in the second step for semi-nested amplification.

3. Results and discussion

3.1. Bacterial composition of the influent water and sewerage biofilms

Microbiological analysis of influent wastewater and biofilms (Table1) indicated high levels of coliform bacteria and faecal enterococci, typical representatives of commensal gut microbiota. It is evident that the highest abundance of all studied bacteria was detected in biofilm from effluent sewerage. Majority of coliform bacteria in biofilm represented *Escherichia coli*, in case of staphylococci prevailed *Staphylococcus aureus.* Effluent biofilm contained more bacteria compared to influent biofilm.

The DGGE showed different bacterial profiles between influent and effluent samples. The influent contained a bigger bacterial diversity than effluent. Such diversity was confirmed also by the sequencing of clones; indeed the effluent microflora included mainly *Pseudomonas* members and few *Acinetobacter* and *Comamonas*. *Pseudomonas* was also the most detected genus in the influent sample, but other bacteria belonged to the genus *Janthinobacterium*, *Acinetobacter*, *Clostridium*, *Achromobacter* and *Aquabacterium* were recognized too.

Table 1: Representation of total selected bacteria in wastewater and municipal sewer biofilms

Bacteria	**Influent water (log CFU/mL)**	**Biofilm influent (log CFU/g)**	**Biofilm effluent (log CFU/g)**
Coliforms	3,9	6,4	7,65
E. coli	2,2	4,2	6,3
Staphylococci	2,82	3,6	4,29
S. aureus	1,9	3	4,15
enterococci	4,74	3,3	4,7

3.2. Prevalence of resistant bacteria in the influent and in sewerage biofilms

With the introduction of antibiotics in the human and veterinary medicine, increases their concentrations in the environment. One of the main sources is wastewater from households, hospitals, and other health care facilities. Subinhibitory concentrations of antibiotics can lead to inductions of bacterial resistance. Our previous study (Birošova *et al*., 2014) showed that wastewater in Slovakia contains predominately ciprofloxacin, clarithromycin and sulphonamides. Wastewater contains also resistant microorganisms, which can spread resistance genes on other bacterial species using horizontal gene transfer. According to this fact resistance against antibiotics of different classes was studied. ß-Lactam (ampicillin) was chosen, because of its frequent prescription in Slovakia (ECDC, 2014). Ciprofloxacin (C), gentamicin (G), tetracycline (T), ampicillin (A) and chloramphenicol (CH) were tested for coliforms. In case of fecal streptococci, susceptibility to ampicillin, ciprofloxacin, gentamicin and vancomycin (V) was studied. Prevalence of ciprofloxacin, gentamicin, tetracycline, chloramphenicol, vancomycin, methicillin (M), erythromycin (E), cephoxitine (Cef), penicillin (P) and ampicillin resistance was studied in *Staphylococcus aureus*. Concentrations of antibiotics were selected according to the resistance breakpoints established by EUCAST (European Committee on Antimicrobial Susceptibility Testing) for EU and by CLSI (Clinical Laboratory Standards Institute) for US. If bacteria are able to grow in the presence of such high antibiotic concentrations, they are considered to be resistant.

From figure 1 and 2 is evident that the highest number of resistant bacteria was observed in effluent biofilm. We have detected also strains that were able to grow on concentrations of antibiotics given by CLSI in this sample. Influent wastewater contained only ampicillin, gentamicin and ciprofloxacin resistant strains. In case of *E.coli* majority of strains were resistant to gentamicin (Fig 1). Majority of strains from biofilm displayed resistance to all tested antibiotics. However, the incidence of nosocomial infections caused by members of the *Enterobacteriaceae* family which produce extended-spectrum β-lactamases and other enzymes capable of hydrolysing β-lactam antibiotics is increasing in the United States and Europe (Schwartz *et al*., 2003).

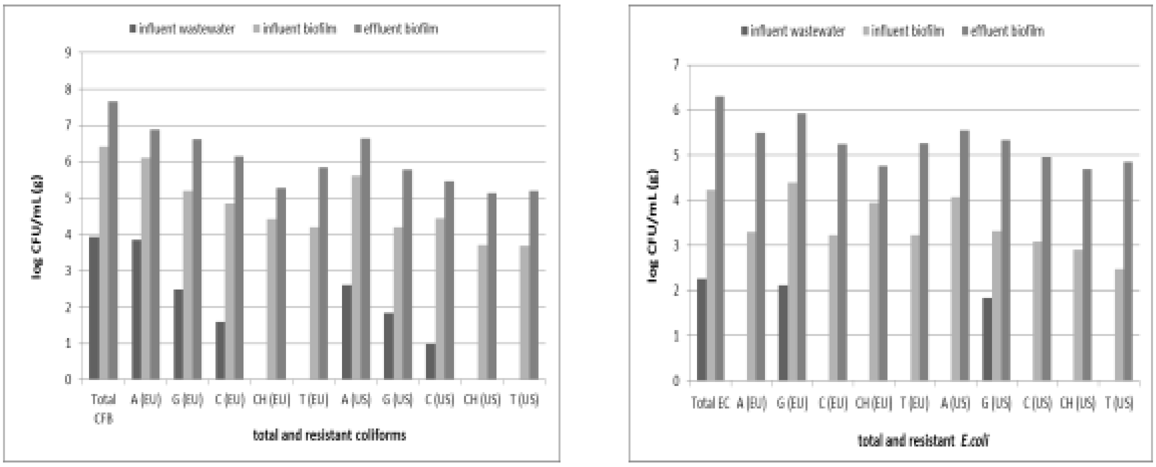

Figure 1: Prevalence of resistant coliform bacteria (CFB) and *Escherichia coli* (EC).

Enterococci from influent wastewater showed resistance only to ampicillin and ciprofloxacin. (Fig.2). On the other hand influent biofilm contained only vancomycin resistant enterococci. In effluent biofilm, majority of strains were resistant to all tested antibiotics. Several reports have also documented the presence, for example, of vancomycin-resistant enterococci (VRE) in the stools of asymptomatic individuals who have neither recently been in hospital nor received antibiotics. Enterococci are also nosocomial pathogens able to cause urinary tract infections, surgical wound infections, endocarditis and bacteremia. Resistance to antibiotics, such as glycopeptides, is a problem in the therapy of these infections (Schwartz *et al*., 2003).The situation was similar in case of *S. aureus* (Fig. 2).The highest number of bacteria resistant to all tested antibiotics was observed in biofilm from effluent sewerage. Methicilin resistant *S. aureus* (MRSA) was observed in all samples. , MRSA is resistant against all other β-lactam antibiotics and only a few antibiotics such as vancomycin or teicoplanin are available for treatment of MRSA infections. Since 1990, has prevalence of these strains increased significantly, and is of special concern because of the limited therapeutic possibilities it entails (Schwartz *et al*., 2003).

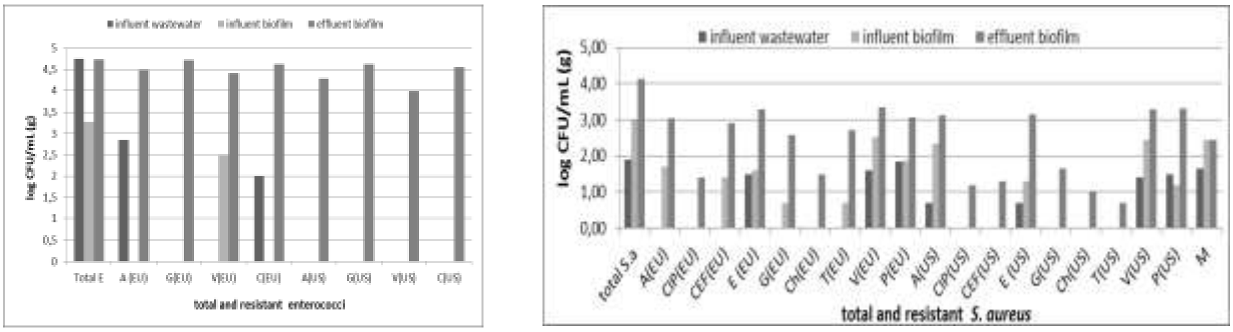

Figure 2: Prevalence of resistant enterococci (E) and *Staphylococcus aureus* (S.a)

4. Conclusions

This investigation evidenced the complementarity of culture-independent and culture-dependent approaches. The use of both strategies permits a better view of the microflora colonizing the wastewater environment. Samples of biofilm contained very high numbers of resistant bacteria comparing to influent wastewater. Bacteria in biofilm from effluent sewerage were more resistant to all tested antibiotics than microorganisms from influent biofilm. Majority of them was able to grow in the presence of high concentrations of antimicrobial compounds established by CLSI. Coliform bacteria were resistant predominantly to ampicillin and gentamicin. Enterococci from influent wastewater were resistant to lower doses of ampicillin and ciprofloxacin, influent biofilm contained only VRE. Majority of enterococci in effluent biofilm were multidrug resistant. Strains of S. aureus in influent wastewater and biofilm were susceptible only to ciprofloxacin and chloramphenicol. High percentage of strains in influent water belongs to MRSA.

ACKNOWLEDGEMENT

This work was supported by the Slovak Research and Development Agency under the contract No. APVV-0122-12

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