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Occurrence of antibiotic resistance genes in sewages of Rostov-on-Don and lower Don River

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Drug resistance has become an extremely serious problem worldwide. Antibiotic resistance genes (ARGs) entering the environment with wastewaters promote replenishment of the resistome of natural microbioms. Distribution of several clinically significant ARGs in wastewaters of Rostov-on-Don (Southern Russia), lower reaches of the Don River and natural waters of the neighboring region was investigated. Metagenomic DNA samples isolated from 250 mL of wastewaters or natural waters and 200 mg of surface sediments were used for the study. Identification of the ARGs was carried out with end-point detection PCR. Presence of *NDM*, *OXA-48*, *CTX-M*, *VanA*, *VanB*, *ErmB*, and *TetM/TetO* genes was detected in urban wastewaters. Samples of wastewater treatment plant (WWTP) sewage were enriched with ARGs in contrast to non-treated wastewaters from the sewage collector. *NDM*, *VanA*, *ErmB*, *TetM/TetO* genes were found only in wastewaters and were absent in samples of natural waters and surface sediments. Only *OXA-48*, *VanB* and *CTX-M* genes were found in natural waters and surface sediments. The described ARGs are quite typical for urban and hospital wastewaters. The target ARGs were detected in the samples connected to the anthropogenous sources of pollution such as Rostov-on-Don municipal WWTP or livestock enterprise effluents.

Keywords: antibiotic resistance; urban wastewaters; natural waters; surface sediments.

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Гены устойчивости к антибиотикам в сточных водах г. Ростова-на-Дону и нижнем течении р. Дон

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Чрезвычайно серьёзной проблемой общемирового масштаба стала лекарственная устойчивость микроорганизмов. Гены устойчивости к антибиотикам (АРГ), попадающие в окружающую среду вместе со сточными водами, способствуют распространению резистентности в природных микробиомах. Исследовано распределение нескольких клинически значимых АРГ в сточных водах г. Ростова-на-Дону, низовьях Дона и природных водах региона. Для исследования использовали образцы метагеномной ДНК, выделенные из 250 мл сточных или природных вод и 200 мг донных отложений. Идентификацию АРГ проводили с помощью ПЦР по конечной точке. Было обнаружено присутствие генов *NDM*, *OXA-48*, *CTX-M*, *VanA*, *VanB*, *ErmB* и *TetM/TetO* в городских сточных водах. Образцы сточных вод очистных сооружений содержали больше АРГ по сравнению с неочищенными сточными водами из сточных коллекторов. Гены *NDM*, *VanA*, *ErmB*, *TetM/TetO* были обнаружены только в сточных водах и отсутствовали в пробах природных вод и донных отложений. В природных водах и донных отложениях обнаружены лишь гены *OXA-48*, *VanB* и *CTX-M*. Описанные АРГ довольно типичны для городских и больничных сточных вод. АРГ были обнаружены в природных пробах, связанных с антропогенными источниками загрязнения, такими как городские очистные сооружения Ростова-на-Дону или сточные воды животноводческого предприятия.

Ключевые слова: устойчивость к антибиотикам, городские сточные воды, природные воды, донные отложения.

Drug resistance has become an extremely serious problem on a world-wide scale. Several decades' application of antibiotics in clinical practice, in veterinary, animal husbandry and aquaculture has led to wide dissemination of antibiotic resistance genes and antibiotic resistant bacteria (ARB). Nowadays bacterial strains carrying several resistance determinants are widespread. A large number of ARGs can be found in hospital, municipal [1] and animal husbandry wastewaters [2]. Actually, ARGs and ARB can change microbial populations and thus must be considered a separate class of important pollutants harmful both for human health and environment.

ARGs are definitely supposed to originate and evolve in natural conditions [3]. It also should be taken into account that ARB pool increases not only due to the mutational processes, but also due to horizontal transfer of genes (HGT) preexisting already in resistomes of various microbic communities [4]. Bacterial mobile elements providing genetic platforms for assembly of multiresistance cassettes participate in this process [5]. Also ARGs transduction by bacteriophages is documented [6].

Water ecosystems are known to have optimum conditions for distribution and acquisition of ARGs by microorganisms [7] due to the continuous inflow of resistant genes from anthropogenous sources. Natural waters are also recognized as the most important pool of accumulation of resistance determinants of anthropogenous origin [8]. Although anthropogenous wastewaters are a constant source of ARGs for the environment, it is important to take into account that natural microbiomes are sources and reservoirs of the genetic material associated with resistance to antibiotics [3]. On the other hand, ARGs dissemination among pathogenic bacteria and environmental bacteria also exists [7]. Thus, the drug resistant bacteria entering the environment with wastewaters (hospital, municipal or agricultural), promote replenishment of the resistome of natural bacterial communities. Besides, they recruit new resistance determinants from these communities [7], promoting increase of the number of drug resistant strains. Studying such circulation of antibiotic resistance is an important task and recently more and more research has been devoted to tackling various aspects of this problem.

In this work we considered distribution of several ARGs common in drug resistant strains of nosocomial origin. Carbapenems, cephalosporins and glycopeptides are widely used in clinical practice now. Besides, tetracycline in large amounts is used in livestock production also as a feed antibiotic.

Materials and methods

Sampling sites. In this research presence of antibiotic resistance genes in wastewaters of Rostov-on-Don (the biggest city in the South of European Russia) and also in water and surface sediments of Lower Don were studied. The Don River is one of the largest rivers in the European part of Russia and the Azov and the Black Sea basin. In its lower reaches the Don River is the main source of water supply for the Rostov region.

Sampling was carried out in 2015–2016. 40 sampling sites were chosen for the study. Sampling sites were situated both upstream and downstream the discharge point of municipal treatment facilities, and also at the small rivers flowing into Don higher up or in the area of the estuary (Fig.).

City sewage was sampled at 9 sites (No. 31–39) in autumn, 2016. WWTP sewage of Rostov-on-Don was sampled in the years 2015–2016 (No. 40–48). Spring water was sampled at 6 sites (No. 23–28) in autumn, 2016. 4 points

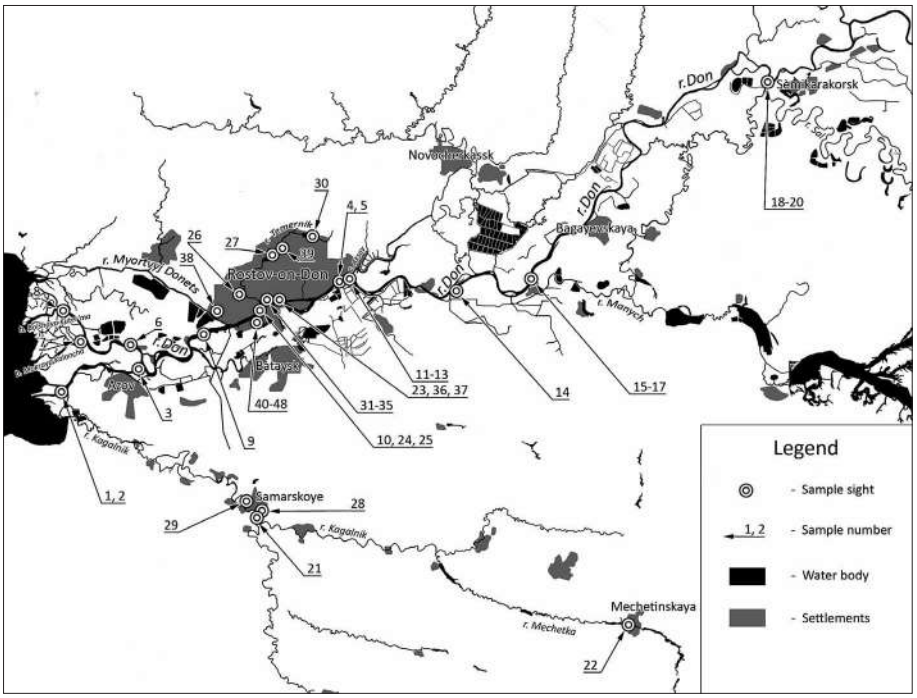

Fig. Sampling sites in the lower reaches of the Don River and in the sewage of Rostov-on-Don

of water and surface sediments selection were located at small rivers of the Lower Don basin (No. 21–22; 29–30). Sampling there was carried out in autumn, 2016. Water and bottom sediments samples of the Don River were taken in autumn, 2015. 7 stations were located on River Don downstream the Rostov WWTP discharge point (No. 1–3; 6–9). 13 stations were located on River Don upstream the Rostov WWTP discharge point (No. 4–5; 10–20).

Samples collection. Sterile plastic bottles were filled with 1 liter of the sampled water each. Water samples were cooled down to +4 °C, taken to the laboratory and processed on the same day.

Bottom sediments samples were selected according to the procedure described in work [9]. Samples were hermetically packed into plastic test tubes and stored at –20 °C before usage in experiments.

Isolation of DNA was carried out according to Galiev and Tsyrulnikov's method modified by us [10]. The short procedure of isolation of total DNA from samples of water and surface sediments is given below.

Isolation of total DNA from water samples. 250 mL water samples were centrifuged for 15 minutes (10000 g, +4 °C). The deposit was suspended in 350 μL of guanidine solution (guanidine HCl 240 mM; phosphate-buffer saline 200 mM; pH 7.0) and 350 μL SDS solution (2% SDS; 500 mM Tris-HCl, pH = 7.9) and then transferred into an screw-cap Eppendorf with 0.2 g glass beads d = 0.5 introduced beforehand. 400 μL of phenol-chloroform mix were added and stirred up on a Mixer Mills MM400 ("Retsch", Germany) mill within 1 minute with the frequency of 30 Hz, then centrifuged for 7 minutes at 14000 g. Water phase was taken, 400 μL of chloroform were added and carefully mixed. Then it was centrifuged like at the previous stage, after that water phase was taken again and 500 μL of isopropyl alcohol were added to it. Everything was kept in the freezer for about 15 minutes, centrifuged for 7 minutes at 14000 g. The deposit was washed out 2 times with 70% ethanol and then dissolved in deionized water.

Isolation of total DNA from samples of surface sediments. For isolation of DNA a frozen surface sediments sample portion of 0,2 g was placed into a 2 mL screw-cap test tube and then glass beads (0.1 g – d = 0.5 mm and 0.1 g – d = 1.0 mm) and ceramic beads (7 pieces of d = 1.0 mm and 3 pieces of d = 2.0 mm) were added. Then 350 μL of guanidine solution (guanidine HCl 240 mM; phosphate-buffer saline 200 mM; pH = 7.0), 350 μl of SDS solution (2%) – Tris-HCl (500 mM, pH = 7.9), and 400 μL of phenol-chloroform mix were introduced into each Eppendorf. The mix was stirred up on a Mixer Mills MM400 ("Retsch", Germany) mill for 15 minutes with a frequency of 30 Hz, then centrifuged for 7 minutes at 14000 g. The water phase was separated, 400 μL of chloroform were added to it and carefully mixed.

Everything was centrifuged just like at the previous stage, then water phase was taken and 500 μL of isopropyl alcohol was added to it. The mix was kept in the refrigerator for 15 minutes, after that centrifuged for 7 minutes at 14000 g. The deposit was washed out 2 times with 70% ethanol and then dissolved in deionized water.

PCR-assay. Amplification reaction was carried out using the T-100 ("Bio-Rad") amplifier and the final volume of reaction mix was 25 μL.

The following sets of PCR-reagents for determination of resistance to antibiotics with electrophoretic end-point detection PCR (NPF Litekh, Russia) were used:

– the Resistance to carbapenems – 1 set, for identification of *VIM* genes;

– the Resistance to carbapenems – 2 set, for identification of *NDM* genes;

– the Resistance to carbapenems – 3 set, for identification of *OXA-48* genes;

– the Resistance to cephalosporins –1 set, for identification of *CTX-M* genes;

– the Resistance to cephalosporins – 2 set, for identification of *MecA* genes;

– the Resistance to glycopeptides set, for identification of *VanA* and *VanB* genes;

– the Eritropol set, for identification of resistance genes to *ErmB erythromycin*;

- the Tetrapol set, for definition of resistance to *TetM/TetO* tetracycline.

The reaction was carried out according to the producer's protocol with the subsequent electrophoretic detection of amplicons. Each reaction included positive and negative controls.

Results and discussion

ARGs analysis results are shown in Table 1.

Seven out of the nine analyzed antibiotics resistance genes have been found in water and surface sediments samples. *NDM*, *OXA-48*, *CTX-M*, *VanA*, *VanB*, *ErmB*, *TetM/TetO* genes have been detected. No samples including wastewaters revealed the presence of *VIM* and

Table

Antibiotic resistance genes in the sewage of Rostov-on-Don and the lower reaches of the Don River

Sample type	Sample No.	Number of samples	Number of samples containing ARGs								
			VIM	*NDM*	*OXA-48*	*CTX-M*	*MecA*	*VanA*	*VanB*	*ErmB*	*TetM/ TetO*
City sewage	31–39	9	–	–	2	1	–	–	3	5	5
WWTP sewage, Rostov-on-Don	40–48	9	–	3	–	–	–	3	5	6	7
Don River water downstream the Rostov WWTP discharge point	1–3, 6–9	7	–	–	–	–	–	–	–	–	–
Don River surface sediments downstream the Rostov WWTP discharge point		7	–	–	–	–	–	–	1	–	–
Don River water upstream the Rostov WWTP discharge point	4–5, 10–20	13	–	–	1	–	–	–	–	–	–
Don River surface sediments upstream the Rostov WWTP discharge point		13	–	–	–	–	–	–	–	–	–
Water from small rivers of the Lower Don basin	21–22, 29–30	4	–	–	–	–	–	–	1	–	–
Surface sediments from small rivers of the Lower Don basin		4	–	–	–	1	–	–	–	–	–
Spring water	23–28	6	–	–	–	–	–	–	–	–	–

Note: the dash "–" means that the gene is not found.

MecA genes within the period of two years of this study.

All wastewater samples contained at least some of the ARGs. Four ARGs families (*NDM*, *VanA*, *ErmB*, *TetM/TetO*) were detected only in wastewaters but not in the samples of natural waters and surface sediments. In 9 wastewater samples taken from municipal WWTP 24 cases of the studied ARGs detection occurred opposed to 16 cases of ARGs detection in 9 samples of wastewaters taken directly from city wastewater sewers. Thus, WWTP sewage is enriched in ARGs compared to sewage from city wastewater sewers. It is of interest that *OXA-48* and *CTX-M* genes were found only in the samples from wastewater sewers, while NDM and VanA were detected only in the samples of waters from WWTP. *ErmB* and *TetM/TetO* genes turned out to be the most widespread in wastewaters. VanB genes proved be the most common among the genes from both wastewaters and natural samples.

ARGs were not very common in natural samples. *VanB* and *OXA-48* were detected in two samples of natural surface water. *CTX-M* genes were detected in one surface sediment sample from small rivers, and *VanB* – in bottom sediments of the Don River downstream of the municipal WWTP discharge point. In all these cases sampling locations were spatially connected with potential anthropogenous sources of ARGs. A discharge point of Rostov municipal WWTP effluents was one such source, another – a livestock farm located in the place of the small rivers Elbuzd and Kagalnik confluence. *OXA-48* marker was detected in the water from the beach of the Alitub village.

It is no surprise that the maximum qualitative and quantitative content of ARGs was observed in wastewaters. It is known that conventional wastewater treatment does not significantly reduce the ARGs concentration and can even sometimes lead to the increase of ARGs concentration in urban wastewaters [11, 12]. WWTPs are a hot spot of horizontal transfer of genetic material. The conditions there are very favorable for exchange of mobile elements of a bacterial genome and amplification and accumulation of a wide range of ARGs and the antibiotic resistant bacteria (ARB) arriving from city collectors of sewage ARGs and antibiotic resistant bacteria (ARB) coming from the city wastewaters collectors. The reason for this is the high content of the extracellular DNA from the destroyed cells and high titers of bacteria and bacteriophages.

It corresponds to the fact that we observed a higher content of ARGs in municipal WWTP effluents compared to the wastewaters sampled directly from the city waste collectors before cleaning. It is substantially connected not only to the continuous receipt of ARGs, but also to the possible high content of mobile elements in bacterial genome, first of all, integrons, in the treated wastewaters [13].

Thus, in the course of collecting, accumulation and preliminary treatment of wastewaters, preceding biological cleaning and disinfection, the quantity of ARGs and ARB can increase dramatically. After final sewage treatment the total amount of ARGs and ARB decreases, as a rule [14, 15]. However, relative frequency of ARGs and ARB in effluents increases simultaneously [13].

Despite the high amount of ARGs in sewage, the number of ARGs significantly reduces as wastewaters enter the environment. So, irrigation with purified wastewaters often doesn't lead to ARGs concentration increase in soils, compared to irrigation with natural waters [16, 17].

The World Health Organization classifies the microorganisms resistant to carbapenems, cephalosporins (especially of the III generation) and to fluroquinolones as the most priority ones. In sewage samples the *NDM* and *OXA-48* genes causing resistance to carbapenems and also *CTX-M* genes providing resistance to cephalosporins were found. Besides, in samples of natural waters *OXA-48* genes, and in bottom sediment samples *CTX-M* genes were discovered. Thus, out of three priority ARG found in sewage, two were also detected in natural samples. It should be pointed out that in both cases not big city wastewaters, but a recreational zone – the beach near the Alitub village (*OXA-48*) and husbandry farm wastewaters (*CTX-M*) were the source.

WWTP dumping into the rivers increases the variety and the ARGs content downstream the dumping place. But as the distance from WWTP increases, the quantity and scope of introduced drug resistance determinants considerably falls, that is typical for both *ErmB* and *Tet* genes. Presence of *TetM* and *TetO* genes is characteristic for municipal wastewaters and animal wastes, thus they are seldomly found in samples of natural waters and soils [18]. Horizontal transfer of *TetO* genes happens less often in comparison to other tetracycline resistance genes because they are less associated with mobile elements in bacterial genomes [19].

Other studied ARGs are also probably eliminated quickly enough in natural microbial

communities as only some of them (*OXA-48*, *CTX-M*, *VanB*) can be found in natural samples, and only in close proximity to the source. Concerning other ARGs which got into the Don and small rivers from wastewaters, concentrations in places of sampling seems to be below the detection limit of the used PCR-kits.

Dissemination of ARGs in WWTP effluents in the environment might be influenced by a range of factors affecting this process. Contamination with antibiotics must obviously facilitate distribution of ARGs [20], but often ARGs distribution is not affected by it [21] . There are other factors that can influence the drug resistance distribution as well. These include microbial community mobilome [22, 23], different types of contaminants, especially heavy metals [24], concentration of biogenic compounds (such as NH_4^+ and PO_4^{3-}), methods of agriculture, water salinity and other factors [23]. Thus, mechanisms of ARGs dissemination modulation in the environment in different conditions requires careful study.

Conclusions

It should be noted that the described ARGs range and distribution are quite typical for urban and hospital wastewaters. Currently, ARGs can be found in surface natural waters and bottom sediments only in close proximity to places of wastewaters dumping and their variety is greatly reduced in comparison with wastewaters. The resistance genes entering the environment with wastewaters definitely pose a certain danger of dissemination of antibiotic resistance in natural microbiomes, but the speed of their elimination from the environment is high enough to prevent wide spreading of ARGs from drains downstream the dumping sites. Recreational zones and insufficiently treated wastewaters of husbandry farms can pose a significant hazard of priority ARGs inflow into the environment.

This research expands the knowledge of ARGs distribution in municipal wastewaters and natural waters in one of the most densely populated southern regions of Russia and, in general, southeastern part of Europe.

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