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Article

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

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Abstract: 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 CTXM 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 municipal WWTP or livestock enterprise effluents.

Keywords: antibiotic resistance; urban wastewaters; natural waters Rostov-on-Don

1. Introduction

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 (multiple drug resistant or polyresistant bacteria) are widespread. Also microorganisms resistant to nearly all of the first line antibiotics (pan-resistant strains) are known [1, 2]. The situation is so tense that there is a real risk of returning to clinical treatment of bacterial infections used before antibiotics discovery [2], and the mankind can find itself in a post-antibiotic era.

A large number of ARGs can be found in hospital [3], municipal [4] and animal husbandry [5, 6, 7] wastewaters. Actually, ARGs and ARB can challenge microbial populations and thus must be

considered a separate class of important pollutants harmful both for human health and environment.

As acquisition of antibiotic resistance by infectious agents significantly complicates patients' treatment, ARGs were mainly studied in a clinical context [8]. At the same time, ARGs are supposed to originate and evolve in natural conditions [9].

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 [10-12]. Bacterial mobile elements providing genetic platforms for assembly of multiresistance cassettes participate in this process [13-16]. Also ARGs transduction by bacteriophages is documented [17, 18].

Most HGT events responsible for the transfer of antibiotic resistance genes occur in human microbiome [19]. Antibiotic usage support ARBs and ARGs import into normal human microbiota and humans become a constant source of drug resistant bacteria in the environment. This process is greatly facilitated by wastewaters.

Hospital wastewaters are especially rich in ARBs and ARGs [3]. At the same time water ecosystems have optimum conditions for distribution and acquisition of ARGs by microorganisms [20] 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 [21-23].

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 [8, 9, 24]. Genetic determinants of antibiotic resistance appeared long before the beginning of antibiotics application and are found in places free from anthropogenous influences. For instance, ARGs aged over 30 000 years were found in permafrost and even in an isolated cave aged more than 4 million years [25, 26]. It should be noted that environmental bacteria produce antibiotics in quantities much lower than the minimum inhibitory concentration [27] and the role they play in natural bacterial communities [28] isn't completely clear. One of the possible explanation of emergence of drugs in subinhibitory concentration might be their role as signaling molecules providing cell-to-cell communication in bacteria, a role important in evolution of antibiotic resistance [29].

On the other hand, ARGs dissemination among pathogenic bacteria and environmental bacteria is also well documented [20, 30). 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 [20, 24], 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.

Despite evident success in this field, it is still not clear how ARGs and ARBs invading with wastewaters are maintained and spread throughout natural water ecosystems and how considerable is the influence of clinically significant drug resistance genes on emergence and distribution of the resistant bacteria associated with human microbiome.

In this work we considered distribution of several ARGs common in drug resistant strains of nosocomial origin. 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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2. Materials and Methods

2.1 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 (Table 1).

Table 1. Description of sampling locations.

№	Sampling site location	Geographical values	Sampling date	Sample type		
				Surface sediments	Water	Wastewaters
1.	«0 kilometre» Don River right bank	47°5.7′N 39°17.5′E	23.09.2015	+	+	
2.	«0 kilometre» Don River left bank	47°5.7′N 39°17.5′E	23.09.2015	+	+	
3.	Don River, 13 km from the estuary	47°12.9′N 39°45.4′E	23.09.2015	+	+	
4.	Don River, 59.5 km from the estuary	47°24.2′N 39°84.9′E	23.09.2015	+	+	
5.	Don River, 59.7 km from the estuary	47°24.3′N 39°85.2′E	23.09.2015	+	+	
6.	Mokraya Kalancha Branch in Dugino village	47°15.6′N 39°43.6′E	24.09.2015	+	+	
7.	Mokraya Kalancha Branch	47°9.8′N 39°20.1′E	24.09.2015	+	+	
8.	Bolshaya Kuterma Branch	47°12.2′N 39°18.1′E	25.09.2015	+	+	
9.	Don River, 0.5 km downstream the sewage of Rostov-on-Don	47°10.3′N 39°34.6′E	25.09.2015	+	+	
10.	Don River, 0.5 km downstream the Temernik River estuary	47°12.4′N 39°41.8′E	26.09.2015	+	+	
11.	0.5 km downstream the Aksai Creek estuary	47°15.0′N 39°52.1′E	26.09.2015	+	+	
12.	Aksai Creek estuary	47°15.1′N 39°52.8′E	27.09.2015	+	+	
13.	Don River, 0.5 km upstream the Aksai Creek estuary	47°14.2′N 39°56.6′E	27.09.2015	+	+	
14.	Don River, Alitub village	47°21.9′N 40°07.6′E	28.09.2015	+	+	
15.	Don River, 0.5 km downstream the Manych River estuary	47°14.7′N 40°14.5′E	28.09.2015	+	+	
16.	Manych River estuary	47° 15.0′N 40°15.1′E	29.09.2015	+	+	
17.	Don River, 0.5 km upstream the Manych River estuary	47°51.3′N 40°15.2′E	29.09.2015	+	+	
18.	Don River, 0.5 km downstream the Sal River estuary	47°32.6′N 40°45.2′E	30.09.2015	+	+	
19.	Sal River estuary	47°31.2′N 40°43.9′E	30.09.2015	+	+	
20.	Don River, 0.5 km upstream the Sal River estuary	47°32.0′N 40°45.2′E	30.09.2015	+	+	
21.	Elbuzd and Kagalnik Rivers confluence (Rostov region, Azov district)	46°55.1'N 39°41.2'E	15.10.2015	+	+	
22.	Mechetka River, Mechetinskaya village	46°76.8′N 40°45.2′E	07.10.2015	+	+	
23.	«Paramonovsky warehouses» spring	47°21.8′N 39°72.7′E	29.09.2016		+	
24.	«Gremuchy» spring (pool)	47°12.2'N 39°41.3'E	11.10.2016		+	
25.	«Gremuchy» spring (pipe)	47°12.2'N 39°41.4'E	11.10.2016		+	
26.	«St. Seraphim Sarovsky» spring	47°22.9′N 39°65.7′E	09.11.2016		+	
27.	«Surb-Khach» spring	47°29.1′N 39°72.4′E	25.10.2016		+	

28.	Spring in Samarskoe village (Rostov region, Azov district)	46°54.5'N 39°41.3'E	15.10.2016		+	
29.	Samarskoe village beach, Kagalnik River (Rostov region, Azov district)	46°56.2'N 39°39.4'E	15.10.2016	+	+	
30.	«Rostov sea» water-storage reservoir	47°30.8′N 39°78.5′E	18.10.2016	+	+	
31.	Sewer on the territory of the food factory	47°21.5′N 39°70.3′E	11.11.2016			+
32.	Sewer on the territory of the food factory	47°21.4′N 39°70.2′E	11.11.2016			+
33.	Sewer on the territory of the market	47°21.7′N 39°71.4′E	17.11.2016			+
34.	Sewer on the territory of the market	47°21.7′N 39°71.3′E	17.11.2016			+
35.	Sewer on the territory of the market	47°21.6′N 39°70.9′E	17.11.2016			+
36.	Storm water drain of the repair plant	47°22.0′N 39°73.9′E	10.11.2016			+
37.	Storm water drain of the repair plant after the oil separator	47°21.9′N 39°73.6′E	10.11.2016			+
38.	Sewer on the territory of the grocery supermarket	47°20.5′N 39°59.8′E	27.10.2016			+
39.	Rostov-on-Don city WWTP	47°29.4′N 39°73.3′E	05.12.2016			+
40.			07.10.2015			+
41.			27.10.2015			+
42.			5.12.2015			+
43.			29.02 2016			+
44.		47°19.1′N 39°68.7′E	30.04.2016			+
45.			31.05.2016			+
46.			10.08.2016			+
47.			19.10.2016			+
48.			12.08.2016			+

City sewage was sampled at 9 sites (no. 31-39). WWTP sewage of Rostov-on-Don was sampled 9 times at the same site (no. 40-48). Spring water was sampled at 6 sites (no. 23-28). 4 points of water and surface sediments selection were located at small rivers of the Lower Don basin (no. 21-22; 29-30). 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).

The detailed information on stations of sampling is provided in Table 1 and Fig. 1.

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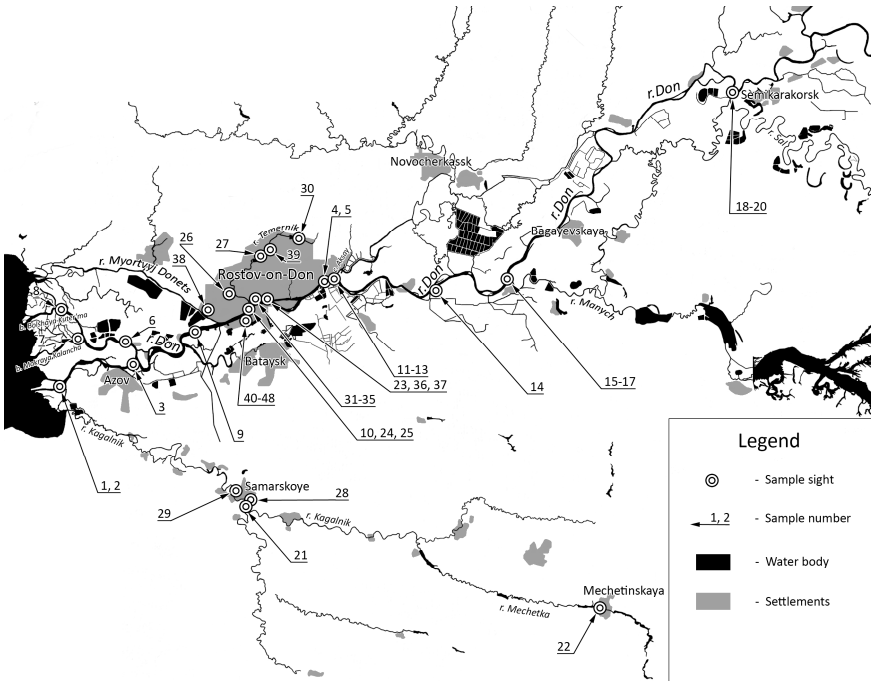

Figure 1. Sampling sites in the lower reaches of the Don River and in the sewage of Rostov-on-Don.

2.2. 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. For the analysis of surface sediments the top two-centimeter layer of deposits was taken. After removal of stones and the plant residues the 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 [31]. The short procedure of isolation of total DNA from samples of water and surface sediments is given below.

2.3. 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 (guanidin 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.

2.4. 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%) -

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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.

2.5. PCR-assay

Amplification reaction was carried out using the T-100 ("Bio-Rad") amplifier and the final volume of reaction mix was 25 µl. Reagents kits by Litekh firm (Moscow) with endpoint detection were used for detection of VIM, NDM, OXA-48, CTXM, MecA, VanA, VanB, ErmB and TetM/TetO genes. 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.

3. Results and Discussion

ARGs analysis results are shown in Table 2. Additional data are given in Figures S1-10 (see the Supplementary Information).

Table 2. 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	1-3, 6-9	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	4-5, 10-20	13	–	–	–	–	–	–	–	–	–

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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	21-22, 29-30	4	–	–	–	1	–	–	–	–	–
Spring water	23-28	6	–	–	–	–	–	–	–	–	–

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

All wastewater samples contained at least some of the ARGs. Four ARG 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 25 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 [32-34]. WWTPs are a hot spot of amplification of ARGs and antibiotic resistant bacteria (ARB) coming from the city waste collectors with wastewaters. 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 [35].

Thus, in the course of collecting, accumulation and treatment of wastewaters, preceding biological cleaning and disinfection, the quantity of ARGs and ARB can increase dramatically. After sewage treatment the total amount of ARGs and ARB decreases, as a rule [36, 37]. However, relative frequency of ARGs and ARB in effluents increases simultaneously [35]. In any case, untreated sewage waters are a bigger threat for the environment [38].

Metagenomic culture-independent methods of research allow to evaluate the total amount of ARGs in the DNA of the studied samples. Treated wastewaters pose a smaller threat of ARGs dissemination in pristine microbial communities. The content of alive ARB decreases in treated wastewaters, but the amount of destroyed bacteria and, respectively, extracellular DNA increases due to disinfection. The role of transformation in resistance distribution in the environment becomes

more significant. At the same time the influence of such effective mechanisms as conjugation and transduction on ARG dissemination decreases.

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 [39-41].

WWTP dumping into the rivers increases the variety and the ARGs content downstream the dumping place [3]. 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 [42]. 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 [43, 44].

Elimination of the other studied ARGs from the environment happens at a lower speed [3]. However, their degradation is likely to be quite fast because only some of them (OXA-48, CTX-M, VanB) can be detected in natural samples taken in the vicinity of their 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 [45, 46], but often ARGs distribution is not affected by it [47, 48]. There are other factors that can influence the drug resistance distribution as well. These include microbial community mobilome [49-51], different types of contaminants, especially heavy metals [49, 52], concentration of biogenic compounds (such as NH_4^+ and PO_4^{3-}) [51, 53], methods of agriculture [51], water salinity [49] and other factors. Thus, mechanisms of ARGs dissemination modulation in the environment in different conditions requires careful study.

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4. Conclusions

The described ARGs range and distribution is quite typical for urban and hospital wastewaters. The resistance genes entering the environment with wastewaters definitely pose a certain danger of dissemination of antibiotic resistance in natural microbiomes. However, the speed of ARG elimination from the environment is high enough to prevent wide spreading of ARGs from drains downstream the dumping sites.

Supplementary Materials: The following are available online, Figure S1: Agarose gel electrophoresis of PCR-amplified OXA-48 genes in samples of natural waters, Figure S2: Agarose gel electrophoresis of PCR-amplified VanA and VanB genes in samples of natural waters, Figure S3: Agarose gel electrophoresis of PCR-amplified CTX-M genes in surface sediment samples, Figure S4: Agarose gel electrophoresis of PCR-amplified VanA and VanB genes in surface sediment samples, Figure S5: Agarose gel electrophoresis of PCR-amplified ErmB genes in wastewater samples, Figure S6: Agarose gel electrophoresis of PCR-amplified CTX-M genes in wastewater samples, Figure S7: Agarose gel electrophoresis of PCR-amplified NDM genes in wastewater samples, Figure S8: Agarose gel electrophoresis of PCR-amplified OXA-48 genes in wastewater samples, Figure S9: Agarose gel electrophoresis of PCR-amplified TetM/TetO genes in wastewater samples, Figure S10: Agarose gel electrophoresis of PCR-amplified VanA and VanB genes in wastewater samples.

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