Antibiotic resistance of microorganisms in agricultural soils in Russia

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Abstract. Antibiotics are medicines that are widely used in livestock production not only for the prevention and treatment of infectious diseases, but also for accelerating the growth of animals. The application of manure for fertilizing agricultural soils leads to the entry into the soil ecosystem not only of the antibiotics themselves, but also the resistance genes to them. In this study, 30 samples of arable soils were tested for the presence of the \textit{tet}(X) gene, which encodes bacterial resistance to antibiotics of the tetracycline group. Using real-time PCR, it was found that 27 out of 30 soil samples contained \textit{tet}(X). 52% of these samples were heavily contaminated, 34% had a medium level of contamination and 14% were slightly contaminated by the resistance gene \textit{tet}(X).

1. Introduction
Almost immediately after the active use of antibiotics for medical purposes in the treatment of human diseases they were also used on a large scale in agriculture [1–3]. The use of antibiotics in animal husbandry aims to achieve the following objectives: disease prevention and treatment and in addition, in sub-therapeutic doses they are used as growth stimulators for animals [4–6]. From the literature it is known that the use of antibiotics in agriculture is quantitatively superior to their use in medicine [1,7]. Antibiotics are substances that have weak adsorption in the body of the animal, so up to 90% of the used dose of the drug is excreted unchanged or as metabolites in the excrement and urine [8–10]. Antibiotics in large quantities therefore enter into the environment when fertilizing soils with manure from farm animals [4]. Antibiotics and their metabolites are able to remain bioactive in soils for several months [1,11]. The mobility and duration of antibiotic residues in the soil depends not only on the physico-chemical properties of the compounds themselves but also on the type of soil, its pH, climatic conditions [7,11]. Many studies indicate the adverse effects of antibiotics on soil microbial communities: the activity of microorganisms is inhibited, changes occur in the ratio of the bacteria and fungi counts, the natural cycle of the elements is disrupted [8,12,13]. All this can ultimately affect the quality and fertility of agricultural soils and harm agricultural production.

In addition to antibiotic themselves, manure contains intestinal microorganisms that are carriers of antibiotic-resistant genes[14]. Therefore, another danger of using manure from animals treated with antibiotics is the spread of resistance genes from the intestinal microflora of livestock to the microorganisms of soil ecosystems [15,16]. Often antibiotic resistance genes are stored in a bacterial cell in the so-called mobile genetic elements – plasmids, transposons, integrons [17]. In the process of
horizontal transfer antibiotic-resistant genes can be rapidly transferred from the donor bacteria to the recipient bacteria, thereby leading to an increase in antibiotic-resistant soil microbial communities [18]. Interestingly, antibiotic resistance genes persist in soils for many years, whereas antibacterial drugs themselves are destroyed within a few months [19].

Recently, the spread of antibiotics and resistance genes in agricultural soils has been of great scientific interest, since it concerns the issue of public health. Genes resistant to antibiotics are able to enter the human body through the food chain (unwashed root crops) and in future they could subsequently hamper the treatment of infectious diseases [20]. Data about agricultural soil pollution by antibiotic resistance genes in Russia is currently not available.

In the present study, 30 samples of agricultural soils selected around the city of Kazan (Republic of Tatarstan) were examined for contamination by the \( \text{tet}(X) \) resistance gene, which is responsible for the destruction of tetracyclines in the bacterial cell. Antibiotics of the tetracycline group are the most commonly used drugs in the animal husbandry of Russia. The Republic of Tatarstan occupies one of the leading places in the agriculture of the Russian Federation, therefore it is important to assess the extent of the spread of antibiotic resistant genes in the arable lands of this region.

2. Material and methods

30 soil samples were taken from cropland around the city of Kazan (N 55°47′, E 49°06′) (Russia). The sampling plots were situated in areas with high agricultural activity (table 1). Soil samples were taken from a depth of 0–20 cm, and cleaned of roots. Immediately after delivery to the laboratory the level of respiratory activity, microbial biomass, soluble organic carbon content and the particle size distribution were estimated as initial parameters of soil samples. To enable further DNA extraction, samples were stored at – 80°C.

The respiratory activity (RA) was assessed according to ISO 14240–1 [21]. The level of microbial biomass (MB) was determined using the method of substrate–induced respiration (ISO 14240–1) [21]. The dissolved organic carbon (DOC) content was determined according to ISO 14235:1998 [22] by a spectrometric method. The particle size distribution of the soil samples was measured on a Microtrack Blue Wave Sample Dell Delivery Controller laser particle analyzer using the Microtrack Blue Wave according to ISO 13320:2009 [23]. Prior to measurement, soil samples were dried and ground to a fine particle state. Extraction of DNA from soil samples was carried out using the FastDNA Spin Kit for Soil kit (MP Bio, Germany) according to the manufacturer’s instructions. DNA purification was carried out using the QIAquick PCR Purification Kit (Quiagen, Germany) according to the manufacturer’s instructions. Detection of the tetracycline \( \text{tet}(X) \) resistance gene was carried out on the real–time cycler using the following primers: \( \text{tet}(X) \)–F GAAAGAGACAACGACCGAGAG and \( \text{tet}(X) \)–R ACACCCATTTGGAAGGCTAAG [24]. The MasterMix reaction mixture contained the following components: DNA template – 1 µl, forward and reverse primers (10 µM) – 0.5 µl each, dNTPs (10 µM) – 2.5 µl, 10x Buffer with SYBR Green – 2.5 µl, MgCl\(_2\) (25 µM) – 2.5 µl, Syn Taq polymerase (5 U µl-1) – 0.2 µl and ddH\(_2\)O – 15.3 µl. Amplification was performed on a BioRadCFX–96 cycler (BioRad, Munich, Germany) using the following temperature program: primary denaturation at 94°C for 3 minutes, then 39 three–step cycles at 94°C for 45 seconds, at 55°C for 45 seconds, and at 72°C for 45 seconds. The level of contamination of soil samples was determined based on the number of cycles of \( C_T \) necessary for fluorescence to reach the threshold level.
Table 1. Soil samples investigated

| Sample number | Coordinates of the soil selection site (N, E) | Information on manure treatment of the soil |
|---------------|-----------------------------------------------|---------------------------------------------|
| 1             | 55.880665, 48.630881                          | cow and chicken manure                      |
| 2             | 55.885069, 48.595461                          | cow and chicken manure                      |
| 3             | 55.867224, 48.620585                          | chicken manure                              |
| 4             | 55.857325, 48.625194                          | chicken manure                              |
| 5             | 55.863052, 48.626243                          | chicken manure                              |
| 6             | 55.863786, 48.585559                          | –                                           |
| 7             | 55.869621, 48.697672                          | chicken manure                              |
| 8             | 55.867390, 48.670235                          | chicken manure                              |
| 9             | 55.886093, 48.665341                          | –                                           |
| 10            | 55.937169, 48.749259                          | chicken manure                              |
| 11            | 55.941670, 48.776962                          | chicken manure                              |
| 12            | 55.865529, 48.817045                          | –                                           |
| 13            | 55.900259, 48.932387                          | chicken and turkey manure                   |
| 14            | 55.893049, 48.866680                          | chicken and turkey manure                   |
| 15            | 55.966047, 49.163583                          | –                                           |
| 16            | 55.895389, 49.288864                          | cow manure                                  |
| 17            | 55.798431, 49.384178                          | goose manure                                |
| 18            | 55.820842, 49.459933                          | chicken and cow manure                      |
| 19            | 55.815556, 49.520443                          | chicken and cow manure                      |
| 20            | 55.802975, 49.580727                          | chicken manure                              |
| 21            | 55.774133, 49.655554                          | chicken manure                              |
| 22            | 55.791594, 49.614571                          | chicken manure                              |
| 23            | 55.792456, 49.586793                          | chicken manure                              |
| 24            | 55.559365, 49.276804                          | chicken manure                              |
| 25            | 55.618271, 49.130810                          | chicken manure                              |
| 26            | 55.553450, 49.203348                          | chicken manure                              |
| 27            | 55.627570, 49.996571                          | –                                           |
| 28            | 55.062182, 50.952912                          | cow manure                                  |
| 29            | 55.175664, 50.781207                          | cow manure                                  |
| 30            | 55.021046, 51.038493                          | cow manure                                  |

3. Results and discussion
The mobility and bioavailability of antibiotics in soil depends on conditions that prevail in the soil (soil type, pH, temperature) [1]. So these factors may influence the development of antibiotic resistance in soil microbial communities under the antibiotic pressure. Furthermore, the fate of antibiotics in the soil may depend on the level of activity of microorganisms, since high activity of microorganisms promotes faster biodegradation, and hence less risk of genes [17]. In addition, the length of time that introduced organisms carrying antibiotic resistance can persist in the soil varies with temperature, moisture, pH, and the indigenous community present [17].

Therefore on the first stage of the study, the agrochemical and microbiological parameters of the investigated soils were analyzed. The measurement results are shown in table 2.
Estimation of dissolved organic carbon (DOC) content in soils showed that 13 of the 30 samples were of low DOC level (samples 1, 2, 5, 12–14, 19, 20, 22–24, 26, 28), 9 samples were of medium DOC level (samples 3, 6, 10, 11, 16, 25, 27, 29, 30) and 8 – of high DOC level (4, 7–9, 15, 17, 18, 21).

The results of the granulometric composition determination of the soils tested showed that the particle size type distribution of 20% of the soil samples was estimated to be sandy loam and 80% of the soil samples – to be silty loam. It is known that in clay soils the antibiotic binds and is inaccessible to microorganisms [3,7]. Studies have shown that under a broad range of environmental conditions tetracyclines (tetracycline, chlortetracycline, and oxytetracycline) can adsorb strongly to clay particles [18]. So among the arable soils investigated soils that were closest to the «sandy loam» type (samples 8, 9, 14, 16, 25) were the most predisposed to dissemination of antibiotic resistance.

As can be seen from the table 2 the arable soils tested characterized by different levels of RA and MB. Thus, these parameters ranged between 0.80 and 5.28 CO₂-C mg g⁻¹ h⁻¹ and between 263.51 and 935.77 µg kg⁻¹, respectively. Since the high microbial activity affects the rate of antibiotic

| Sample number | DOC, mg g⁻¹ | Soil type     | RA, CO₂-C mg g⁻¹ h⁻¹ | MB, mg kg⁻¹ |
|---------------|-------------|---------------|----------------------|-------------|
| 1             | 0.07        | Silty loam    | 1.27                 | 281.52      |
| 2             | 0.09        | Silty loam    | 1.92                 | 264.82      |
| 3             | 0.11        | Silty loam    | 1.73                 | 263.51      |
| 4             | 0.44        | Silty loam    | 1.23                 | 240.4       |
| 5             | 0.05        | Silty loam    | 1.19                 | 382.2       |
| 6             | 0.14        | Silty loam    | 0.80                 | 298.06      |
| 7             | 0.45        | Silty loam    | 0.96                 | 280.97      |
| 8             | 0.43        | Sandy loam    | 2.48                 | 442.16      |
| 9             | 0.30        | Sandy loam    | 2.39                 | 479.68      |
| 10            | 0.11        | Silty loam    | 2.90                 | 578.88      |
| 11            | 0.12        | Silty loam    | 2.09                 | 557.48      |
| 12            | 0.09        | Silty loam    | 1.25                 | 373.33      |
| 13            | 0.04        | Sandy loam    | 2.29                 | 323.67      |
| 14            | 0.06        | Sandy loam    | 1.17                 | 314.52      |
| 15            | 0.24        | Silty loam    | 1.88                 | 392.00      |
| 16            | 0.13        | Sandy loam    | 2.32                 | 334.96      |
| 17            | 0.27        | Silty loam    | 2.51                 | 450.09      |
| 18            | 0.25        | Silty loam    | 2.07                 | 368.70      |
| 19            | 0.02        | Silty loam    | 2.02                 | 466.97      |
| 20            | 0.09        | Silty loam    | 1.70                 | 476.78      |
| 21            | 0.21        | Silty loam    | 1.77                 | 361.86      |
| 22            | 0.04        | Silty loam    | 2.57                 | 367.99      |
| 23            | 0.08        | Silty loam    | 1.96                 | 402.28      |
| 24            | 0.04        | Silty loam    | 2.43                 | 642.09      |
| 25            | 0.11        | Sandy loam    | 2.06                 | 706.22      |
| 26            | 0.09        | Silty loam    | 2.66                 | 635.13      |
| 27            | 0.15        | Silty loam    | 3.13                 | 608.00      |
| 28            | 0.06        | Silty loam    | 4.61                 | 706.84      |
| 29            | 0.11        | Silty loam    | 3.49                 | 732.85      |
| 30            | 0.11        | Silty loam    | 3.54                 | 935.77      |
biodegradation, soils with a low level of microbial activity (samples 1–7, 12, 14, 20, 21) may be susceptible to the spread of resistant genes [18].

Many studies indicate a link between the application of manure contaminated by antibiotics and the presence of antibiotic resistance in treated arable soils [25–27]. So, in the next stage the contamination of the soils by the tet(X) gene was analyzed by real–time PCR. The results are shown in table 3. To assign a level of contamination to arable lands by the tet(X) resistance gene, the CT values obtained by real–time PCR reaction were ranked in three categories. Score 0 was assigned to the samples without contamination by tet(X) gene, scores 1, 2 and 3 were given to samples with low, middle and high contamination, respectively. As can be seen from the table 3, 27 of the 30 agricultural soils tested contained the tetracycline resistant gene tet(X). Out of 27 contaminated agricultural soils, 52% were heavily polluted, 34% showed medium level pollution and 14% were slightly polluted by tet(X) gene. According to the literature a high abundance of tet(X) gene has been found in manure and manure-treated soils by other authors [28–31].

Table 3. Contamination of soil samples by tet(X) gene

| Sample number | C_r value | Contamination level by tet(X) gene | Sample number | C_r value | Contamination level by tet(X) gene |
|---------------|-----------|-----------------------------------|---------------|-----------|-----------------------------------|
| 1             | 25.78     | 3^d                               | 16            | 25.65     | 3                                 |
| 2             | 28.38     | 0^a                               | 17            | 25.42     | 3                                 |
| 3             | 25.29     | 3                                 | 18            | 26.45     | 2                                 |
| 4             | 25.73     | 3                                 | 19            | 27.59     | 1                                 |
| 5             | 25.51     | 3                                 | 20            | 26.55     | 2                                 |
| 6             | 25.87     | 3                                 | 21            | 26.61     | 2                                 |
| 7             | 26.86     | 2^c                               | 22            | 26.08     | 2                                 |
| 8             | 28.38     | 0                                 | 23            | 27.42     | 1                                 |
| 9             | 24.61     | 3                                 | 24            | 26.33     | 2                                 |
| 10            | 23.64     | 3                                 | 25            | 32.11     | 0                                 |
| 11            | 25.39     | 3                                 | 26            | 26.91     | 2                                 |
| 12            | 25.61     | 3                                 | 27            | 26.02     | 2                                 |
| 13            | 27.11     | 1^b                               | 28            | 26.19     | 2                                 |
| 14            | 27.64     | 1                                 | 29            | 25.45     | 3                                 |
| 15            | 25.55     | 3                                 | 30            | 24.84     | 3                                 |

Contamination of soil samples by tet(X) gene:

- a – no contamination
- b – low contamination
- c – middle contamination
- d – high contamination

Despite the absence of any kind of manure treatments for soil samples 6, 9, 12, 15, 27 for at least the last 10 years, these soils had middle and high levels of pollution by resistance gene tet(X). Probably the presence of antibiotic resistance in these soils can be associated with the previous practice of fertilizing by manure from tetracycline medicated animals. Many studies also point to the fact that antibiotic resistant genes are persist in agricultural soil for a long time even when there is no continued application of manure [32–34].

In terms of the manure type that leads to the greatest antibiotic resistance gene contamination, chicken manure was the most problematic. Previously, we demonstrated that chicken manure is more polluted by the tet(X) resistance gene compared with cow, pig, goat and rabbit manures [35].
Conclusion
This study has effectively demonstrated that the agricultural soils sampled around Kazan are at potential risk of antibiotic resistance dissemination because of their agrochemical and biological properties. Among the soils investigated, more than 90% were contaminated by the tetracycline resistant gene tet(X). The results obtained confirm the data that antibiotic resistant genes spread in the soil through the introduction of manure and remain in the soil despite the termination of the practice of annual manure spreading.

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