Evaluation of antibiotic resistance of Enterobacteriaceae bacteria isolated from water objects of the north-west of the Murmansk region

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Abstract. Multidrug antimicrobial resistance among different species of the family Enterobacteriaceae remains a major problem worldwide. In this paper, we report the data on antimicrobial susceptibility of 145 isolates of Enterobacteriaceae collected in 14 water environments of the north-west of the Murmansk region in autumn 2016. The number of Enterobacteriaceae in the examined rivers was rather low. Only the concentration of enterobacteria exceed the regulations in water samples from lake Kuetsjarvi. The detected isolates were assigned to eight species of enterobacteria. The most abundant species were Enterobacter spp. (26.9%) and Citrobacter freundii (25.3%). Isolates of Escherichia coli were not detected. Most of the isolates (96.6%) were multi-drug resistance (MDR). Notably, 16.6% of the all isolates were categorized as possible extensively drug-resistant (XDR). Almost all isolates were showing high level resistance to κ-lactams, especially to cefepime and imipenem. Most effective antimicrobial agents were tobramycin and norfloxacin.

1. Introduction
The problem of the development and spread of antibiotic resistance among microorganisms is becoming more and more relevant. In recent decades, a significant increase in the resistance of bacteria to antimicrobial agents has been registered widely. Such a significant spread of antibiotic resistance is a natural response of microorganisms to the usage of antibiotics in medicine, veterinary medicine and agriculture. Antibiotics create selective pressure, contribute to the selection, survival and spread of resistant strains of microorganisms [1]. In addition, the spread of multidrug-resistant bacteria has been observed almost everywhere in recent years. The emergence of "super" resistant strains has led the World Health Organization (WHO) to recognize the problem as global.

Antimicrobial resistance is of great socio-economic importance and is seen as a threat to national security in the developed world [2]. In recent years, opportunistic microorganisms have played an important role becoming the main cause of opportunistic diseases in conditions of increased anthropogenic load and environmental pollution [3]. Members of the Family Enterobacteriaceae play
a special role among these microorganisms. Therefore, the study of the spread of antibiotic resistance among these bacteria is very relevant and significant. It is well known that the spread of antibiotic resistance is local, regional and national. Different areas have their own characteristic spectra of antibiotic resistance. Features of the structure of aquatic ecosystems and their natural processes, anthropogenic influences, in particular, the introduction of antimicrobials used to treat people, as well as antibiotics used in agriculture for the treatment and stimulation of animal growth, have an impact on the formation of these spectra.

All household effluents from the surrounding area fall directly or indirectly (through the soil) into surface waters. Coliform bacteria are characterized by accumulation in the external environment, because the rate of their reproduction and preservation of viability exceeds the rate of cell death [4]. Water bodies can be considered as "reservoirs" of the majority of variants of enterobacteria which meet in the considered territory, including differing on level of resistance to antimicrobial agents. Such studies have not previously been conducted in the Murmansk region. The objective of this study is to assess the resistance of members of the Family *Enterobacteriaceae* isolated from water bodies of the North-West of the Murmansk region to antibacterial drugs.

2. Materials and methods

2.1. Water samples collection

Water sampling for bacteria isolation of Enterobacteriaceae family was performed in accordance with the General requirements of GOST R 31942-2012. Water samples were taken from a depth of 10-15 cm from the surface into sterile 500 ml glass vials.

Water samples were taken in September 2016 from 14 surface water bodies of the North-West of the Murmansk region: rivers Pechenga, Shuonijoki, Kuvernerinjoki, Kotsel'joki, Kolosjoki, Namajoki, Menikkajoki, Laukkujoki, Sejgijoki, Kornetijoki, Nautsijoki, Paz, and from the lake Kuetsjarvi and the stream flowing into the lake Luchlumpolo.

2.2. Bacterial counts, isolation and species identification

We used the membrane filtration to count the number of member of Enterobacteriaceae. First, water samples were filtered through 0.45 μm membrane filters (Vladisart, Russia). The filters were placed on Endo agar. The plates were incubated for 24-48 h at 37°C. Species identification of isolates was carried out using a test-system for rapid biochemical identification of enterobacteria "Rapid Entero 200 M" (Institute of epidemiology and microbiology named after Pasteur, St.-Petersburg, Russia), which is based on microvolume technology using liquid differential medium.

2.3. Antibiotic susceptibility test.

The susceptibility of the Enterobacteriaceae isolates to antibiotics was assessed using the disk diffusion technique on Mueller-Hinton agar (HiMedia, India) Strains were tested against different antimicrobial agents: ampicillin (10 μg), ampicillin-sulbactam(20 μg/10 μg), ticarcillin -clavulanic acid (75 μg/10 μg), (75 μg), cefazolin (30 μg), cefuroxime (5 μg), ceftriaxone (30 μg), cefepime (30 μg), imipenem (10 μg), gentamicin (30 μg), amikacin (30 μg), tobramycin (5 μg), ciprofloxacin (5 μg), levofloxacin (1 μg), norfloxacin (2 μg), ofloxacin (5 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), nitrofurantoin (300 μg), and tetracycline (30 μg). The data were interpreted using the EUCAST criteria [5] and the software package of the analytical control system of antibiotic resistance “WHONET” 5.6.

3. Results

The members of the family *Enterobacteriaceae* were found in all 14 surveyed surface water bodies of the North-West of the Murmansk region. The surveyed water bodies were characterized by a low abundance of these bacteria in water samples. Thus, Enterobacteria concentrations ranged from 0.71 in samples collected from of the rivers Namaiki to 123.4 CFU/100 mL in samples collected from...
Paatsjoki river near in the area of HPP "Hevoskoski"), the average is 21.8±6.30 CFU/100 ml. The content of enterobacteria in the waters of lake Kuetsjarvi was significantly higher (1064.7±158.3 CFU/100 ml). This is explained by the location of urban settlement Nickel – the administrative center of the Pechenga district on the shore of this lake. Thermotolerant coliform bacteria (indicators of recent pollution) were not found.

145 isolates representing the family Enterobacteriaceae were isolated from 14 analyzed surface water bodies. The species composition of the studied isolates is presented in table 1. Enterobacter spp. and Citrobacter freundii isolates were found most frequently in aqueous samples (26.9% and 25.3%, respectively). Other frequently encountered species were Kluyvera ascorbata (11%) and Providencia alcolifaciens (11%). Escherichia coli isolates were not found in water bodies during this period.

| Isolate                  | Number (%) |
|-------------------------|------------|
| Enterobacter spp        | 39 (26.9)  |
| Citrobacter freundii    | 37 (25.3)  |
| Kluyvera ascorbata      | 16 (11.0)  |
| Providencia alcolifaciens| 16 (11.0) |
| Hafnia alvei            | 14 (9.7)   |
| Citrobacter koseri      | 11 (7.6)   |
| Klebsiella oxytoca      | 8 (5.5)    |
| Klebsiella mobilis      | 4 (2.7)    |
| **TOTAL**               | 145        |

All analyzed enterobacteria isolates were resistant to one or more antimicrobial agents (table 2). According to international criteria [6], 140 (96.6%) isolates possessed multiple resistance phenotype (MDR) — non-susceptibility to at least one agent in three or more antimicrobial categories. Moreover, bacteria that do not have MDR were found only among representatives of Enterobacter spp. and H. alvei, isolated from Kolosjoki river. These isolates were resistant to separate β-lactam antibiotics, one of them was also insensitive to gentamicin. Compared with the research results 2007-2009 [7], a significant increase in the frequency of isolates with MDR phenotype noted in this study.

24 (16.6%) of the Enterobacteriaceae isolates had an extreme resistance phenotype (XDR) - resistance to all drugs except one or two categories of antimicrobials. Isolates with XDR phenotype were found most frequently among the Citrobacter koseri (54.5%) and Providencia alcolifaciens (37.5%). This is due to their natural resistance to many antibiotics [8,9]. Most of the XDR isolates retained sensitivity only to drugs of the fluoroquinolone group (79.2% of the strains were sensitive to levofloxacin, and 70.8% to norfloxacin,70.8% to ofloxacin) and aminoglycosides – torbamycin (70,8%) and gentamycin (62.5%).

| Isolate                  | Number of resistant isolates to 1 and more antibiotics (%) | Number of MDR isolates (%) | Number of XDR isolates (%) |
|-------------------------|-------------------------------------------------------------|----------------------------|---------------------------|
| Enterobacter spp        | 39 (100)                                                   | 35 (89.7)                  | 5 (12.8)                  |
| Citrobacter freundii    | 37 (100)                                                   | 37 (100)                  | 4 (10.8)                  |
| Kluyvera ascorbata      | 16 (100)                                                   | 16 (100)                  | 1 (6.3)                   |
| Providencia alcolifaciens| 16 (100)                                                  | 16 (100)                  | 6 (37.5)                  |
| Hafnia alvei            | 14 (100)                                                   | 13 (92.9)                 | 1 (7.1)                   |
| Citrobacter koseri      | 11 (100)                                                   | 11 (100)                  | 6 (54.5)                  |
| Klebsiella oxytoca      | 8 (100)                                                    | 8 (100)                   | 1 (12.5)                  |
| Klebsiella mobilis      | 4 (100)                                                    | 4 (100)                   | 0 (0)                     |
| **TOTAL**               | 145 (100)                                                  | 140 (96.6)                | 24 (16.6)                 |
The results of the stability assessment of all Enterobacteriaceae isolates as well as individual species of this family are presented in table 3. The analyzed isolates were the least sensitive to β-lactams among all studied antimicrobial groups. Insensitivity (resistance or moderate resistance) to ampicillin was found in more than 70% of isolates. Slightly fewer cultures (62.1% and 54.5%, respectively) were resistant to ampicillin/subactam and ticarcillin-clavulanate, and the highest number of isolates sensitive to these drugs was among Enterobacter spp. and K. mobilis. Considering the resistance to cephalosporins, it immediately draws attention to the fact that the stability of the entire sample of isolates increases from II generation to IV. Moreover, if only 25.5% of isolates are resistant to cefepime (II generation)(among the representatives of the genus Klebsiella resistance variants are not detected), the resistance to cefepime (IV generation) is 87.6%. About 90% of C. koseri, K. oxytoca, Pr. alcolifaciens and Kl. ascorbate is immune to this drug. Resistance to imipenem is in the range from 50 to 93.7% of the analyzed isolates.

Among aminoglycosides the least effective drug was amikacin (45.5% of resistant cultures). Resistance to gentamicin was about 25%. Torbacycine was the most effective drug amongst all the antibiotics in relation to the entire sample of isolates (19.3%) and the most overwhelming C. kosery (9.1% resistant variants), C. freundii (18.9%) and K. ascorbata (12.5%).

On average, about 30% of isolates were resistant to fluoroquinolones. The only exceptions were K. ascorbate, P. alcolifaciens and H. alvei, more than half of which have been insensitive to ciprofloxacin. Resistant variants to norfloxacin were not found (0%) among Klebsiella. All isolates of K. mobilis is sensitive to ofloxacin.

Resistance to trimethoprim-sulfamethoxazole ranged from 12.5 to 54.5%. K. oxytoca isolates were the most susceptible to the action of this antibiotic. The effect of nitrofurantoin strongly depends on the species of the isolate. Only about a quarter of the isolates of K. oxytoca, K. ascorbate, P. alcolifaciens had resistance to this antibiotic. Whereas, 75% of the K. mobilis isolates, and 81.2% of P. alcolifaciens isolates showed insensitivity to nitrofurantoin.

Insensitivity to chloramphenicol and tetracycline showed 49.7% and 50.3%, respectively, of all enterobacteria isolates. The level of resistance of Enterobacteriaceae species to these two antimicrobials seemed very similar.

4. Conclusions
The results of this study indicate a wide spread of resistance to most antimicrobial drugs among the members of the family Enterobacteriaceae, isolated from surface water bodies of the North-West of the Murmansk region. Almost all isolated isolates were multidrug-resistant. Thus, the detected microorganisms, being opportunistic bacteria, may pose a threat to the health of people who live or visit the surveyed water bodies. Monitoring and control of the spread of antibiotic resistance genes in the environment is increasingly seen as an environmental problem, especially in Northern aquatic ecosystems, which are very vulnerable to a variety of impacts.

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| Antimicrobial agent | Enterobacter spp. | Citrobacter freundii | Citrobacter koseri | Klebsiella oxytoca | Klebsiella moebii | Kluyvera ascorbata | Providencia alcalifaciens | Haemophilus alvei | TOTAL |
|---------------------|------------------|---------------------|-------------------|------------------|-----------------|------------------|-------------------------|-----------------|-------|
| Ampicillin          | 69.2             | 75.7                | 54.5              | 87.5             | 75.0            | 81.3             | 81.2                    | 85.7            | 75.2  |
| Ampicillin/Sulbactam| 56.4             | 64.9                | 42.7              | 62.5             | 25.0            | 50.0             | 75.0                    | 71.4            | 62.1  |
| Ticarcillin/Clavulanic acid | 28.2      | 70.3                | 54.5              | 50.0             | 56.3            | 75.0             | 64.2                    | 54.5            |       |
| Cefazolin           | 28.2             | 21.6                | 27.3              | 0.0              | 0.0             | 43.8             | 25.0                    | 28.6            | 54.5  |
| Cefuroxime          | 51.3             | 51.4                | 81.8              | 62.5             | 0.0             | 43.8             | 50.0                    | 35.7            | 66.2  |
| Ceftriaxone         | 69.2             | 64.9                | 72.7              | 25.0             | 75.0            | 68.8             | 75.0                    | 64.2            | 50.3  |
| Cefepime            | 79.5             | 86.5                | 90.9              | 100.0            | 75.0            | 100.0            | 93.7                    | 85.7            | 87.6  |
| Imipenem            | 74.4             | 70.3                | 90.9              | 87.5             | 50.0            | 62.5             | 93.7                    | 71.4            | 75.2  |
| Amikacin            | 43.6             | 43.2                | 63.6              | 50.0             | 25.0            | 31.3             | 43.7                    | 64.2            | 45.5  |
| Gentamicin          | 17.9             | 24.3                | 36.4              | 12.5             | 25.0            | 25.0             | 37.5                    | 21.4            | 24.1  |
| Tobramycin          | 17.9             | 18.9                | 9.1               | 25.0             | 25.0            | 12.5             | 25.0                    | 28.6            | 19.3  |
| Ciprofloxacin       | 23.1             | 29.7                | 27.3              | 37.5             | 25.0            | 62.5             | 56.2                    | 57.1            | 37.2  |
| Levofoxacin         | 23.1             | 37.8                | 27.3              | 62.5             | 25.0            | 37.5             | 18.7                    | 42.8            | 32.4  |
| Norfloxacin         | 12.8             | 32.4                | 27.3              | 0.0              | 0.0             | 37.5             | 6.3                     | 14.3            | 20.0  |
| Ofloxacin           | 17.9             | 24.3                | 27.3              | 12.5             | 0.0             | 37.5             | 37.5                    | 42.8            | 26.2  |
| Trimethoprim/Sulfamethoxazole | 33.3      | 43.2                | 54.5              | 12.5             | 25.0            | 25.0             | 43.7                    | 35.7            | 36.6  |
| Nitrofurantoin      | 43.6             | 59.5                | 45.5              | 25.0             | 75.0            | 25.0             | 81.2                    | 28.6            | 48.3  |
| Chloramphenicol     | 41.0             | 54.1                | 72.7              | 37.5             | 25.0            | 43.8             | 75.0                    | 35.7            | 49.7  |
| Tetracycline        | 46.2             | 56.8                | 45.5              | 87.5             | 25.0            | 37.5             | 62.5                    | 35.7            | 50.3  |