RESEARCH ARTICLE **(Open Access)**

# Rivers, hotspots for Antimicrobial Resistance: ESBL Producing Escherichia coli in Shkumbini River, Albania

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## Abstract

It has been well documented that aquatic environment such as lakes as rivers has served, serve and will serve as common reservoirs for different organisms, with a significant proportion of water samples tested positive for ESBL E. coli. Our objective has been to explore the diversity, occurrence and to have one screen in phenotypic as well as molecular traits of ESBL E. coli, by concentrating in isolating and investigating quantity of them from the sample collected. Water samples were taken from Shkumbini river which flows through different municipalities in an area that includes some of the main cities of Albania such as Librazhd and Elbasan and is characterized by intense anthropogenic activity and agriculture. Eight sampling sites were selected based on a combination of factors, including their proximity to potential sources of contamination, their accessibility, and their representation of different areas of Shkumbini River. Fifteen E. coli strains identified as ESBL-producers were isolated from 12 (75%) of 16 water samples. According to antimicrobial susceptibility testing, the highest resistance frequency was observed to streptomycin (93.33%), ampicillin (93.33%), and gentamycin (73.33%). Considering the content of antibiotic resistance genes, three different β-lactamase gene families (bla OXA-1, bla TEM, bla CTX-M1) were detected in ESBL-producing E. coli strains. Further research is needed to better understand the prevalence of these genes in the river and the potential risks they may pose to human and animal populations. Specifically, future studies should aim to identify the sources of contamination and assess the efficacy of current water treatment and sanitation measures in reducing the spread of these genes.

**Keywords:** ESBL E. coli Albania, antimicrobial resistance, river water

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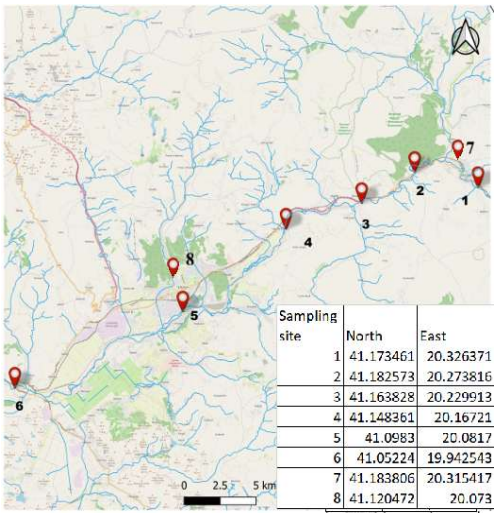
## 1. Introduction

We have to remain deeply concerned about the limited development in pharmaceutical line for new treatments targeting drug-resistant infections, especially those caused by gram-negative bacteria. Some infections are now resistant to all available antibacterial therapies and despite caution optimism regarding a few preclinical agents there is an urgent need for drugs effective against resistant's organisms (1) At the same time the environmental spread of antibiotics resistance genes (ARGs) poses a big concern for human and animal health, yet the role of bacteriophages in mediating ARG transfer thought transduction remains underexplored. Recent findings indicate that relying solely on ARG deduction rates and abundance does not adequately capture bacteriophage driven dissemination, emphasizing the value of combined indices such as occurrence rate, absolute phage abundance and phage to bacteria ratios for environmental risk assessment (2). Bacterial ARGs can be mobilized by mobile genetic elements and growing evidence suggest that bacteriophages and phage-redivided particles may significantly contribute to ARG dissemination across environmental system. ARGs are frequently detected in both bacterial and bacteriophage fractions of aquatic environments and their long-term persistence is crucial factor enabling successful horizontal transfer (3). Most of bacteria form biofilms on biotic or abiotic surfaces and these structured communities are particular concern because they reduce antibiotic effectiveness and facilitate the acquisition and spread of ARGs. Environmental biofilms are now recognized as key hotspots for ARG dissemination, as horizontal gene transfer-mediated by mobile genetic elements and occurring more frequently in biofilms than planktonic cells (4). The extensive use of antibiotics in human medicine, veterinary and agriculture leads to their continues release into environment along with antibiotic-resistant bacteria (ARB) and ARGs, particularly from wastewater. Wastewater treatment plants are recognized as major hotspots for dissemination of antibiotic resistance (5). The effect of sewage overflow in urban rivers, and the agricultural influences from river water suggest one upstream (6). Antibiotic resistance is recognized as a major threat to public health. Bacteria utilize a range of mechanisms to evade the effects of antibiotics leading to challenges in clinical infection treatments. These range from non-specific processes including increased efflux pump activity or down regulation of porin channels, to the production of enzymes that specifically target and inactivate antibiotics (7). To assess public health risks from environmental exposure to Extended-Spectrum β-Lactamases (ESBL)-producing bacteria, it is necessary to have insight in the proportion of relative harmless commensal variants and potentially pathogenic ones (which may directly cause disease) (8). As bacteria continue to adapt to the presence of antibiotics through the acquisition of antibiotic resistance genes (ARGs) via mobile genetic elements (MGEs), available antibiotics are becoming less effective. Due to the growing limitation of treatment options, older antibiotics such as colistin, which can cause negative side effects are being employed to treat infections caused by bacteria that are resistant to last resort antibiotics (9).

## 2. Materials and methods

### *2.1 Description of the Study Location and Sample Collection*

Sampling was conducted along the Shkumbini River a primary Albanian river spanning 181 km with a mean annual discharge of 61 m³/s, it passes through varied urban centers, industrial area and intensive agricultural areas reaching the Adriatic Sea (figure 1). Two sampling were carried out, in April 2023 and August 2023, at eight sites (S1-S8), yielding a total of 16 water samples. Sites were selected according their proximity to potential contamination sources and were distributed by following the WHO Tricycle Protocol for global surveillance of ESBL-producing E. coli, for assess the impact of high density the sapling sites were established upstream and downstream (10) of Elbasan (population proximally 232 580 (11)). Sites coordination are given at figure 1. At each site, 500 ml of surface water was collected at depth of approximately 30 cm in sterile containers, transported at 4°C and processed within 24h of collection.




| Sampling site | North | East |
|---|---|---|
| 1 | 41.173461 | 20.326371 |
| 2 | 41.182573 | 20.273816 |
| 3 | 41.163828 | 20.229913 |
| 4 | 41.148361 | 20.16721 |
| 5 | 41.0983 | 20.0817 |
| 6 | 41.05224 | 19.942543 |
| 7 | 41.183806 | 20.315417 |
| 8 | 41.120472 | 20.073 |

**Figure 1.** Location and map of the Shkumbin river. The sampling sites are indicated by their numbers

### *2.2 Identification and microbial isolation*

Isolations was performed by membrane filtration following the WHO Tricycle Protocol to have alignment with international surveillance practice (10). For each water samples, (100 ml) was filtered on vacuum through 0.45 µm cellulose nitrate membrane filters (Millipore, Burlington, MA, USA). To enumerate total *E. coli*, filters membrane was transferred to Tryptone Bile X-glucuronide (TBX) chromogenic agar, to recover presumptive ESBL-producing E. coli, parallel filters were placed on TBX supplemented with 4 µg/mL cefotaxime (Sigma-Aldrich, St. Louis, MO, USA), incubated at 37°C for 24h, and green-blue colonies were counted as presumptive E. coli. Counts were expressed as CFU/100 ml (table 1).

Up to thre presumptive colonies per cefotaxime positive sample were randomly collected and purified by subculture on MacConkey agar. Phenotypic ESBL producing was confirmed by double disk synergy test (DDST) on Mueller-Hinton agar (Merck, Darmstadt, Germany), with an amoxicillin-clavulanate disk 30 µg placed centrally and cefotaxime 5 µg, ceftazidime 10 µg and cefepime 30 µg disks positioned 20 mm away in accordance with EUCAST guidelines (12). Enhancement of the inhibition zone of cephalosporin towards the clavulanate disk was recorded as positive result.

### *2.3 Antimicrobial susceptibility Testing and resistance phenotyping*

All positive and confirmed ESBL-producing E. coli isolates were tested against seven antimicrobial agents representing five antimicrobial categories: ampicillin (AMP; 10 µg; penicillin), imipenem (IMP; 10 µg) and meropenem (MEM;10 µg; carbapenems), gentamicin (CN; 10 µg) and streptomycin (S; 10 µg; aminoglycosides), ciprofloxacin (CIP; 5 µg; fluoroquinolones) and tetracycline (TE;30 µg). Susceptibility was determined by the Kirby-Bauer disk diffusion method on Mueller-Hinton

agar (MHA). Susceptibility data were interpreted following European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (13), the exception of tetracycline, which was evaluated according to Clinical and Laboratory Standards Institute (CLSI) criteria (14). Quality control was maintained using *E. coli* ATCC25922 as a reference strain. Isolates exhibiting non susceptibility to at least one agent in three or more antimicrobial categories were classified as multidrug resistant (MDR) (15).

### *2.4 Detection of Resistance Genes and Phylogenetic Typing*

ESBL-Ec strains were characterized with respect to their phylogenetic group and the genetic basis of ESBL production. For this purpose, genomic DNA was extracted from overnight cultures using a commercial bacterial DNA extraction kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and stored at −20 °C until use.

Phylogenetic grouping of ESBL-Ec isolates was performed using a multiplex PCR method as previously described (16). The presence of major β-lactamase gene families associated with ESBL production in ESBL-Ec isolates was investigated by multiplex PCR. Detection of seven β-lactamase gene families, including $bla_{\text{CTX-M-1}}$ group, $bla_{\text{CTX-M-2}}$ group, $bla_{\text{CTX-M-9}}$ group, $bla_{\text{CTX-M}}$ group 8/25, $bla_{\text{OXA-1-like}}$, $bla_{\text{SHV}}$, and $bla_{\text{TEM}}$, was achieved using specific primers and conditions as previously described (17).

## 3. Results

### *3.1 Prevalence of ESBL-EC*

A total of 15 isolates exhibiting ESBL production were isolated from water samples from Shkumbini river. Those ESBL-Ec was found in 75 % (12/16) of water samples taken from all sampling points.

| | Sample | **TBX** | **TBX +** |
|---|---|---|---|
| Sampling time | | **E. coli** | **E. Coli** |
| Apr-23 | **S 1** | **130** | **3** |
| | **S 2** | **2** | **1** |
| | **S 3** | **200** | **6** |
| | **S 4** | **180** | **3** |
| | **S 5** | **174** | **1** |
| | **S 6** | **280** | **25** |
| | **S 7** | **206** | **0** |
| | **S 8** | **148** | **0** |
| Aug-23 | **S 1** | **70** | **1** |
| | **S 2** | **63** | **4** |
| | **S 3** | **60** | **2** |
| | **S 4** | **78** | **5** |
| | **S 5** | **200** | **8** |
| | **S 6** | **148** | **11** |
| | **S 7** | **20** | **0** |
| | **S 8** | **40** | **0** |

**Table 1:** Counts of presumptive E. coli recovered by membrane filtration from Shkumbini River water samples on TBX agar without and with cefotaxime (4 µg/mL), by sampling site (S1–S8) and campaign (April and August 2023).

### *3.2 Antibiotic Resistance Phenotypes*

All 15 ESBL- producing isolates were tested against seven antimicrobial agents representing five categories. The highest resistance frequencies were recorded to streptomycin (93.33%, 14/15), ampicillin (93.33%, 14/15), and followed gentamycin (73.33%, 11/15). Resistance was therefore most frequent among aminoglycosides and penicillins (figure 2).

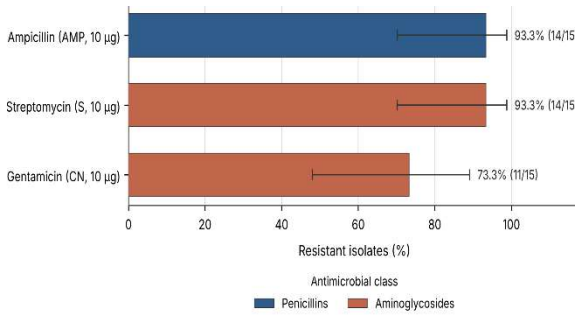



**Figure 2:** The three most frequent antimicrobial resistances among ESBL-producing E. coli isolates (n = 15) from the Shkumbini River, Albania.

### *3.3 Distribution of ß-lactamase Genes*

Among the seven ß-lactamase gene families screened by multiplex PCR, tree was detected. At least one target gene was identified in 4 of 15 isolates (26.67%). The bla$_{CTX-M-}$1 group was the most frequently detected family (2/15; 13.3%), followed by bla$_{TEM}$ and bla$_{OXA-1-}$ (1/15 each; 6.7%). In the remaining 11 isolates (73.3%), none of the seven targeted families was detected despite a positive double disk synergy test.

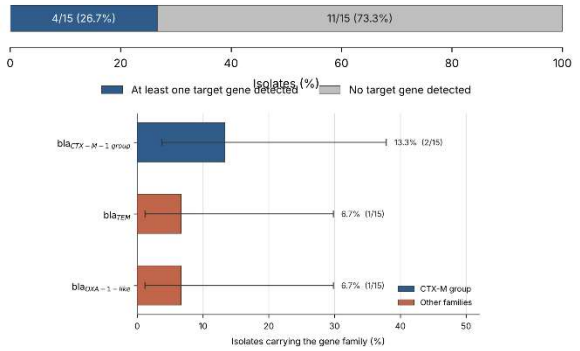



**Figure 3:** β-lactamase genes in ESBL-producing E. coli from the Shkumbini River (n = 15). (a) Detection frequency of each gene family; (b) overall PCR screening outcome. Error bars: 95% CI.

## 4. Discussion

The study conducted for the first time in the laboratory of Veterinary faculty of Tirana, agriculture university of Tirana and giving first comprehensive phenotypic and molecular characterization of ESBL-Ec of surface waters taken from Shkumbini river and based on the technique used in another laboratory in Greece that gave the first results about of this topic for Albania and Shkumbini river samples. Detection of ESBL-Ec in 75% of the analyzed samples indicates a contamination spread on aquatic ecosystem with antimicrobial resistant bacteria. these findings support different hypothesis that surface waters serve as reservoir for antimicrobial resistance determinates and resistant bacterial population. Similar high detection rates have been reported in river systems worldwide, that suggest anthropogenic activities play a pivotal role in environmental dissemination of this *Enterobacteria* (19) (18). Nearly all isolates demonstrate resistance from ampicillin, and a significant of them showed resistance to third generation cephalosporins, reflecting selective pressure by β-lactam antibiotic use. This high prevalence to ampicillin and third generation of cephalosporins among *E. coli* isolates in Shkumbini river is a matter of prefund clinical concern and epidemiological concern, because ampicillin is one of most used antibiotics widely to treat various infections in humans and animals (20).The molecular characterization of ESBL genes isolates in this study reveals a clear predominance of blaCTX-M-1 group, detected. CTX-M type enzyme is currently most widespread ESBLs globally and are frequently associated with environmental and clinical *E. coli* strains, in same time are primary drivers of ESBL dissemination in aquatic ecosystems (21) (22).

## 5. Conclusions

ESBL-producing *E.coli* were collected from 12 of 16 water samples collected along the Shkumbini river in 2023, with the highest propotions of cefatoxime-resistant E.coli downstream of Elbasan. The isoletes was frequently resistant to ampicillin, streptomycin and gentamicin, meanwile carbapenemes retained full activity and, bla CTX-M-1 was the most frequently detected ß-lactamase gene family.This study demonstrate that the Shkumbini river represent an important environmental reservoir for antimicrobial resistance bacteria, high prevalence, genetic heterogeneity and diverse resistance gene shows a contamination from multiple anthropogenic sources. Despite data provided this study is limited because of small number of isolated analyzed. Further large-scale research and longitudinal investigates are requested to have a more accurate characterize of antimicrobial resistance in these aquatic ecosystems. Those finding rise the concern and the importance of implementing integrated environmental surveillance programs.

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**Conflicts of Interest:** The authors declare that they have no conflicts of interest.

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