Proceedings

Spread of Antibiotic Resistance in Aquatic Environments: *E. coli* as a Case Study [†]

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Abstract: The aim of this study was to gather information on the spread of antibiotic resistance in *Escherichia coli* isolates from wells, boreholes and untreated drinking water in islands of Greece. We analyzed for antibiotic resistance 235 *E. coli* strains isolated from untreated drinking water of small rural communities, and ground water from 4 islands. Resistance was tested against Norfloxacin, Ciprofloxacin, Levofloxacin, Amoxicillin and Cefaclor. More than half (54.9%) were resistant to at least one of the antibiotics tested. Of these 26.3% showed multiple resistance (to two or more antibiotics). Strains from drinking water sources were overall more sensitive. Frequent resistance was observed for Amoxicillin (38.3%) and Levofloxacin (28.5%), low for Norfloxacin (5.5%).

Keywords: quinolones; cephalosporins; drinking water; ground water; intermediate resistance

1. Introduction

Antibiotic resistant microorganisms have become, the last few decades, a challenge to infection control. Antibiotics appear with increased frequency to be less effective against microorganisms and, depending on the pathogen, the incurable infections are threatening to become an everyday phenomenon resulting in increased fatality. The current resistance to antimicrobial drugs has been estimated to account for 700,000 deaths a year. If the antimicrobial resistance increases it is predicted to account for 10,000,000 deaths/year by 2050 [1].

In the environment antibiotic resistant bacteria accumulate, arising either by accidental mutation, by mutation in humans or animals under antibiotic treatment selection or are naturally occurring environmental bacteria who, under selective pressures (an evolutionary response resulting from natural selection) have developed resistance for their own protection against competitors [2]. Resistance genes from environmental bacteria can be transferred to clinically important pathogens via horizontal gene transfer, pointing to the fact that environment is an important component of spread of antibiotic resistance to humans.

The amount of antibiotics consumed by humans is phenomenal. In livestock, too, excessive amounts are used for growth promotion and as feed additives, as well as in veterinary medicine. Antibiotic resistant bacteria from both sources are excreted through waste and are dispersed into aquatic environments either directly or by effluents of Waste Water Treatment Plants discharged in surface waters. In many countries deposition of manure or sludge on land as a fertilizer is a widespread practice. So is the use of WWTP effluents for irrigation and urban applications. Underground aquifers can be affected indirectly. The demand for water has led to use of WWTP effluent for replenishing aquifers, so antibiotic resistant bacteria, which have survived treatment processes, can be injected in aquifers directly. Aquaculture practices, the administration of antibiotics

to fish for disease prevention or therapeutically, charges the aquatic environment with excess antibiotics leading to possibility of antibiotic resistant bacteria in aquaculture products.

Drinking water is a particularly important milieu for dissemination of such bacteria, which could infect the general population producing serious Pubic Health consequences. Several investigations have been conducted on the occurrence of antibiotic resistance and multiresistance in drinking water. As early as 1981 in an extensive investigation of drinking water in the USA, 33.9% of the 2653 heteroptophic gram positive and gram negative bacteria isolated were found multiresistant, and of these 2.9% were resistant to all 5 antibiotics tested [3]. Massa et al. [4] isolated heterotrophic resistant bacteria from bottled mineral water in Italy, of which 51% were multiresistant. More recently heterotrophic bacteria from drinking water in the USA, before and after treatment, were found resistant to amoxicillin (3.0–39.6%), to ciprofloxacin (0.2–13.4%), to rifampicin (10.8–82.1%) and to lesser extend to other antibiotics [5]. In a U.K. investigation 77.7% of heterotrophic bacteria from tap water were resistant to at least one antibiotic, 40.6% had multiple resistance. Amoxicillin resistance was the most prevalent (64.9%), 8.8% were detected to resist in ciprofloxacin [6]. In Greece 91.3% of antibiotic resistant gram negative bacteria isolated from drinking water (mainly enterobacteriaceae, vibrionaceae and *Pseudomonas* sp.) were multiresistant. The highest resistance was to cephalotin, ampicillin and carbenicillin. No resistance was detected for quinolones, aminoglycosides, imipenem, aztreonam, ceftazidime or cefoperazon [7]. All 32 injured coliforms recovered from drinking water in Argentina, *Klebsiella oxytoca*, *Enterobacter aerogenes* and *Enterobacter cloacae*, were resistant to aminopenicillin-sulbactam and nitrofurantoin while they were sensitive to the quinolones tested (norfloxacin and ofloxacin) [8].

Patterns of antibiotic resistance of *E. coli* strains isolated from drinking water have been described in very few countries. In India all *E. coli* strains recovered from chlorinated drinking water of a tropical city were multiresistant to at least 4 antibiotics, 100% were resistant to nalidixic acid, 95% to amoxicillin, 25% to norfloxacin, 10% were resistant to ciprofloxacin [9]. In a study in China 49.5% of isolates from drinking water sources before treatment were resistant to at least one antibiotic. 24.0% exhibited multiple resistance. Amongst the 18 antibiotics tested, the strains showed the highest resistance to amoxicillin, ciprofloxacin and levofloxacin [10]. In Pakistan in a study examining resistance patterns to 2 antibiotics, 29.0% of *E. coli* from tap water were resistant to kanamycin and ampicillin [11]. In another investigation 63.0% of *E. coli* isolates from drinking water were multiantibiotic resistant, 92.6% were resistant to nalidixic acid, 88.9% to ampicillin, 40.7% to ceftriaxone, 37.0% to ciproflaxin [12]. In Canada 10.5% of the *E. coli* isolated from private drinking water sources were resistant to minimum one antibiotic, 3.7% to more than 3 antibiotics [13].

Not many studies are published investigating the problem of antibiotic resistance in bacteria from ground water used for drinking purposes. In 1988 Amundson et al. [14] reported that in well water 45% of total coliforms and 16% of fecal coliforms were multiresistant, with resistance most commonly to ampicillin, cephalotin, nitrofurantoin and tetracycline. In a study of rural untreated groundwater supplies, 100% of the non-coliform bacteria and 87% of the coliforms isolated were resistant to at least one antibiotic of the 16 antibiotics tested. Novobiocin, cephalotin and ampicillin were the antibiotics toward which the highest resistance was directed. Multiresistant were 60% of the coliforms—of which 14% were *E. coli*. Above 95% of non-coliforms exhibited multiple resistance patterns [15]. In Egypt, in a study recently published, three *E. coli* strains were isolated from ground water, all of them multiresistant [16].

Should humans contact—or consume—antibiotic resistant microorganisms and submit to infection, the possibility of successful antibiotic treatment is severely reduced. The widespread enrichment of environmental waters with antibiotic resistant bacteria and genes make it plausible that such bacteria find their way into drinking water sources, survive treatment processes and create public health issues. In Greece the wide consumption of antibiotics and the development of resistant bacterial strains to even the most advanced antibacterial drugs [17] is the cause of grave concern. In this study we used *Escherichia coli* strains isolated from water sources (groundwater and untreated drinking rural community water) to address the questions: 1. Which is the sensitivity of these faecal organisms to 5 antibiotics. 2. What is the resistance patterns of simple- versus multi-resistance.

2. Materials and Methods

2.1. Site Description

Fresh water samples were collected from four Greek islands (Figure 1). The drinking water was untreated and came from small rural communities; the ground water was collected from boreholes and wells from the same islands. It is used mostly for irrigation.
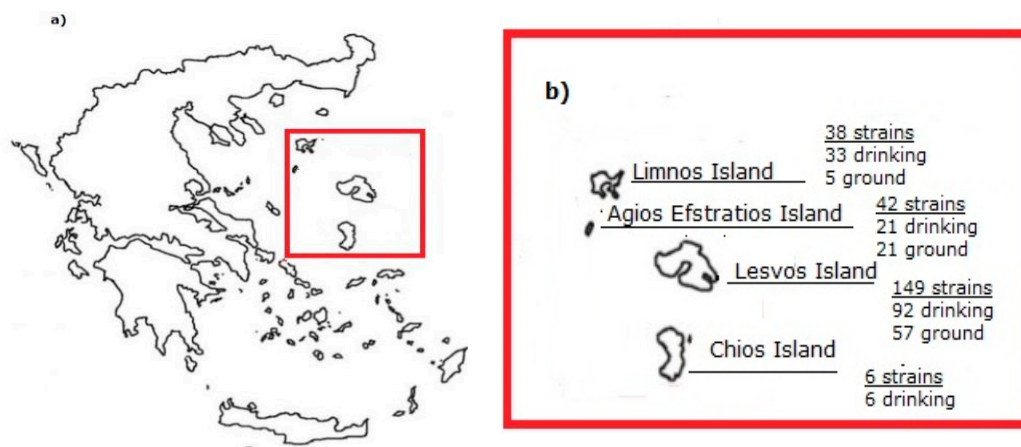

Figure 1. Sampling areas: (**a**) map of Greece; (**b**) the four Greek islands and number of isolated *E. coli* strains from each.

2.2. Sample Collection

Sampling was conducted according to ISO 19458:2006throughout 2017. The samples were collected in 500 mL dark glass sterile bottles and carried to the Laboratory on ice. Upon arrival their temperature was recorded, and was between 2.0–8.0 °C. They were processed within a maximum of 24 h from collection.

2.3. Escherichia coli Isolation

235 strains of *E. coli* were isolated by membrane filtration (ISO 9308-1:2014). 158 strains came from the drinking, untreated water, 77 strains were from ground water sources. Membranes were placed on Chromocult Coliform Agar (Merck cat.no. 1.10426.0500). β-D-galactosidase positive colonies (dark-blue to violet color) were transferred and incubated on Tryptic Soy Agar (Merck cat.no. 1.05458.0500). Oxidase negative colonies were transferred in Tryptone Water (Merck cat.no.VM 610494.346) to perform Indole test (ISO 8199:2005). Colonies positive for indole and for β-D-galactosidase tests were considered as *Escherichia coli*.

2.4. Antibiotic Resistance

The resistance of the 235 *E. coli* strains was tested against five antibiotics widely used in medicinal and veterinary practice: three quinolones (Norfloxacin-NXN, Ciprofloxacin-CIP, Levofloxacin–LVX) one cephalosporin (Cefaclor-CEC) and one penicillin (Amoxicillin-AMX) Becton Dickinson. Amoxicillin is a relatively 'old' antibiotic, came into use in 1972, and belongs to the penicillin group. Quinolones are amongst the more recent additions to the antibiotic family, and are considered very effective. Norfloxacine was introduced in the 80's, Ciprofloxacine in the 90's, Levofloxacine around 2000. Escherichia coli strain CRM09001L was used for quality control of the screening protocol. The bacteria were tested by the Kirby–Bauer disk diffusion susceptibility test [18]. Interpretation of results was conducted based on the EUCAST Testing Guidelines—European Committee on Antimicrobial Susceptibility [19], and strains were classified as 'resistant', 'intermediate' or 'sensitive' (Table 1). Strains exhibiting "intermediate" resistance patterns were classified as "resistant" for the purposes of this study.

Table 1. Concentration (µg) of antibiotics and diameter (mm) of inhibition zones.

Antibiotic	Concentration	Inhibition Zone mm		
		Resistant	Intermediate	Sensitive
Amoxicillin AMX	25 µg	≤1.3	1.4–1.6	≥1.7
Cefaclor CEC	30 µg	≤1.4	1.5–1.7	≥1.8
Norfloxacin NXN	10 µg	≤2.0	2.1–2.4	2.5–3.1
Ciprofloxacin CIP	5 µg	≤2.4	2.5–2.7	2.8–3.5
Levofloxacin LVX	5 µg	≤.5	2.6–2.8	2.9–3.6

3. Results

Of the 235 *E. coli* strains we examined more than half (54.9%) were resistant to at least one of the antibiotics tested. Of these 26.3% showed multiple resistance (to two or more antibiotics). 13.6% were resistant to two antibiotics, 9.8% were triply resistant, 0.8% were quardriply resistant and 2.1% were resistant to the five antibiotics tested (Table 2).

Table 2. *Escherichia coli* strains resistant to one or more antibiotics.

Resistance to:	Drinking Water	Ground Water	Total
	Strains (%) [1]	Strains (%) [1]	Strains (%) [1]
1 antibiotic	41 (25.9)	26 (33.8)	67 (28.5)
2 antibiotics	23 (14.6)	9 (11.7)	32 (13.6)
3 antibiotics	11 (7.0)	12 (15.6)	23 (9.8)
4 antibiotics	-	2 (2.6)	2 (0.8)
5 antibiotics	5 (3.1)	-	5 (2.1)
Total strains	80 (50.6)	49 (63.7)	129 (54.9)

[1] Percentage of the total (235) number of strains examined.

Strains from drinking water sources were overall more sensitive, although by little, exhibiting lower multiple resistance patterns (24.7% as opposed to 29.9% of the ground water strains). More specifically, 80 of the 158 *E. coli* strains from drinking untreated water (50.6%) were resistant to at least 1 antibiotic. Half of these (41–25.9%) were resistant to only one antibiotic, five (3.1%) were resistant to all 5 antibiotics tested. Ground water *E. coli* had higher percentages of resistance: of the 77 strains examined 49 (63.7%) showed resistance to one or more antibiotics. Although the percentage of resistant strains was higher, more than half of them (26–33.8%) were resistant to only one antibiotic, only two (2.6%) were resistant to 4 antibiotics, none resisted all 5 tested (Figure 2).

Amoxicillin was the antibiotic with the highest resistance response (38.3%) followed by Levofloxacin (28.5%), Cefalosporin, (18.3%), Ciprofloxacin (8.5%) and Norfloxacin (5.5%). In drinking and in ground water the patterns of resistance were the same: in Amoxicillin the highest resistance, in Norfloxacin the lowest. There was no antibiotic, of those tested, to which 100% of the isolates were susceptible (Table 3).
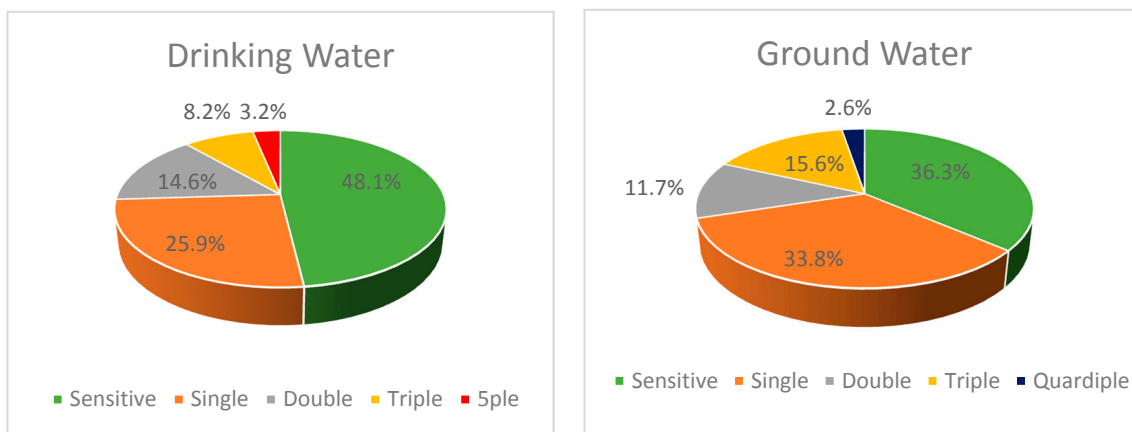
Figure 2. Frequency of resistance of *E. coli* strains isolated from drinking and ground water to 5 antibiotics.

Table 3. Resistance of *E. coli* strains to 5 antibiotics.

	AMX	CEC	NXN	CIP	LVX
	Number [1]—%	Number [1]—%	Number [1]—%	Number [1]—%	Number [1]—%
Drinking	54–34.2%	29–18.4%	9–5.7%	11–7.0%	42–26.6%
Ground	36–46.8%	14–18.2%	4–5.2%	9–11.7%	25–32.5%
Total	90–38.3%	43–18.3%	13–5.5%	20–8.5%	67–28.5%

[1] Number of resistant strains; AMX—amoxicillin; CEC—cefaclor; NXN—norfloxacin; CIP—ciprofloxacin; LVX—levofloxacin.

4. Discussion

The results of our investigation showed that resistance to antibiotics is widespread amongst *E. coli* that proliferate untreated water used for drinking purposes in small rural communities in four Greek islands. According to several authors [9–14] the situation is not overall different in other countries. In every research project looking for *E. coli* or faecal coliforms in drinking water there were strains exhibiting resistance to antibiotics. The differences were in the percentages which ranged from 100% resistant strains in India [9], to 10.5% in Canada [13]. In our research we found 50.6% to be resistant to at least one antibiotic. Our observations in multiresistance patterns are not similar with these of other research teams. We had multiresistance in 24.7% of our strains from drinking water sources. The findings of other authors ranged from 100% in India [9] to 3.7% in Canada [13]. The higher percentages in resistant strains were reported from countries where the use of antibiotics in humans and animals is less strictly controlled.

In ground water 63.6% of our strains showed resistance to one or more antibiotics. Of these 29.9% were multiresistant. This is not different from results elsewhere. In an early study in the US [14] it was reported that 16% of faecal coliforms isolated from well water was multiresistant. In a study published 26 years later [15] 87% of coliform bacteria isolated from ground water in the US were multiresistant. Of these 14% were *E. coli*. In Egypt [16] 100% of *E. coli* isolated from groundwater exhibited multiple resistance. All investigators report single resistance to be more often met than multiple resistance. It is worth noting that the more antibiotics the strains are tested against, the more multiresistant findings.

Because of the very small number of relevant studies internationally, and to the fact that in each study a different set of antibiotics is examined, it is not possible to perform a comparison as to resistance patterns of waterborne *E. coli* strains. Amoxicillin is an antibiotic against which most bacteria from our study had resistance genes (34.2%), 95.0% resistance was documented in India [9]. Ciprofloxacin and Levofloxacin, second and third generation quinolones, are antibiotics heavily

prescribed, and this reflects in the fact that we observed high resistant *E. coli* strains (11.7% and 32.5%) to these two antibiotics in ground water.

The existence of *E. coli* in drinking water is in itself disturbing. In countries with high standards of living it is attributed mostly to the fact that there exist many families who live in farms and remote locations, where there is no mains connection and water used for drinking comes from private, untreated sources. The existence of so many antibiotic resistant strains of this bacterium is alarming, given the fact that not only *E. coli* itself could under certain circumstances, cause illness, but even more because of the ability of the resistance genes to transfer amongst microorganisms, transferring the resistance genes to pathogens. Our work demonstrates that untreated drinking and ground water can contribute to the spreading of antibiotic resistance to the general population, posing a serious challenge to infection control.

Author Contributions: M.A.E. conceived and designed the experiments; M.B. performed the experiments under the su-pervision of E.K.; M.A.E. and M.B. analyzed the data; all three authors contributed in the writing of the paper, read and approved the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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