



Ambiente & Água - An Interdisciplinary Journal
of Applied Science

ISSN: 1980-993X

ambi-agua@agro.unitau.br

Universidade de Taubaté
Brasil

Lösch, Liliana Silvina; Alonso, José Mario; Merino, Luis Antonio
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subtropical region of Argentina
Ambiente & Água - An Interdisciplinary Journal of Applied Science, vol. 3, núm. 2, 2008, pp. 28-36
Universidade de Taubaté
Taubaté, Brasil

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Occurrence of antimicrobial-resistant *Enterobacteriaceae* in water from different sources in a subtropical region of Argentina

Liliana Silvina Lösch¹; José Mario Alonso²; Luis Antonio Merino³

Instituto de Medicina Regional, Universidad Nacional del Nordeste, Chaco, Argentina
E-mail: ¹silvinalosch1@argentina.com; ²jmalonso@gigared.com; ³lamerino@gigared.com

ABSTRACT

In order to determine the occurrence of antimicrobial-resistant *Enterobacteriaceae*, 506 samples of drinking water, surface water, and ground water were studied in the province of Chaco, Argentina. One hundred and ninety one isolates of *Enterobacteriaceae* Family members were obtained. The most frequent specie was *Escherichia coli* and it showed the highest rate of acquired resistance, being the β -lactams the most affected antibiotics. The findings of the present work suggest that the occurrence of antimicrobial-resistant bacteria in aquatic ecosystems of Chaco may contribute to the environmental dissemination of antibiotic resistance.

Keywords: antimicrobial resistance; aquatic environments; drinking water; *Enterobacteriaceae*; ground water; surface water.

Ocorrência de *Enterobacteriaceae* resistentes aos antimicrobianos nas águas de diferentes fontes em uma região subtropical de Argentina

RESUMO

A fim de determinar a ocorrência de *Enterobacteriaceae* resistentes aos antimicrobianos, 506 amostras de águas de bebida, águas superficiais, e águas subterrâneas foram estudadas na província do Chaco, Argentina. Cento e noventa e um isolados de membros da família *Enterobacteriaceae* foram obtidos. A espécie mais freqüente foi *Escherichia coli* e mostrou uma taxa mais elevada de resistência adquirida, sendo os β -lactamicos os antibióticos mais afetados. Os resultados do trabalho atual sugerem que a ocorrência das bactérias resistentes aos antimicrobianos em ecossistemas aquáticos do Chaco pode contribuir para a disseminação ambiental da resistência antibiótica.

Palavras-chave: resistência antimicrobiana; ambientes aquáticos; água de bebida; *Enterobacteriaceae*; água subterrânea; água superficial.

1. INTRODUCTION

Antimicrobial resistance has an important impact on public health policies and it involves an increasing number of bacterial species and resistance mechanisms and It has been observed that the biggest increment in antibiotic-resistant bacteria occurred in those countries where antibiotics are extensively used for prevention or treatment of microbial infections in humans as well as in veterinary medicine (Kümmerer, 2004; Junco-Díaz et al., 2006).

Besides of the consequences for the human health, the concern of the scientific community on this topic did increase in the last years due to the geographical expansion of superficial and deep water sources contamination with resistant bacteria and with residues of antimicrobial agents or their metabolites. This situation should call the attention of scientists because antibiotics, on the contrary to other chemical compounds, exercise a direct action on bacteria and can act as persistent pollutants by its continuous emission to the different aquatic compartments (Hirsch et al., 1999; Alonso et al., 2001).

Antimicrobial surveillance programs provide important information on the development of bacterial resistance mechanisms in different geographical regions. Data concerning these mechanisms and patterns of antimicrobial resistance allows the implementation of changes in antimicrobial prescribing practices and infection control interventions (Jones and Masterton, 2001)

Most of the works about surveillance on antimicrobial resistance have been carried out in bacteria isolated from clinical samples; however, studies should also be expanded to those bacteria recovered from environmental samples in order to evaluate their role as possible reservoir of resistance genes and their capacity to transfer them to human pathogenic organisms. (Alonso et al., 2001; Harakeh et al., 2006).

Enterobacteriaceae members are broadly distributed in the environment and they are etiological agents of a great number of infectious diseases (Schreckenberger et al., 1999). Antimicrobial-resistant enterobacteria have been isolated from a variety of sources (Boon et al., 1999; Córdoba et al., 2001; Sayah et al., 2005), but up to now there is not any data about this topic in a subtropical area characterized by a great diversity of aquatic ecosystems.

The aim of the present work was to study the occurrence of antimicrobial-resistant *Enterobacteriaceae* in different sources of water in a subtropical region of Argentina, in order to determine their possible role as reservoir of antimicrobial resistance.

2. MATERIALS AND METHODS

2.1. Study area

The survey was performed in the province of Chaco (Northeast of Argentina), a plain region characterized by an average annual temperature of 20.5°C, a mean annual rainfall of 1350 mm, and with 1 million inhabitants approximately. The province has two well-differentiated geographical regions: the western region is dry with only one river, without lakes or lagoons, and less populated than the eastern region, which is highly irrigated by rivers and present many big lagoons; the population of this last area is about 700,000 people.

2.2. Sampling collection

A total of 506 water samples were taken from both geographical regions, including surface waters (rivers and lagoons), ground water (perforations deeper than 6 meters), and drinking water (tankers and pipeline). Distribution of samples is shown in Table 1.

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Table 1. Locations and number of water samples (n) taken in the Province of Chaco (Northeast of Argentina).

Region	Rivers		Lagoons		Groundwater		Drinking water		Total	
	location	n	location	n	location	n	location	n	locations	samples
Western	Bermejo	3	Mistolalar	3	Avia Teraí	4	Corzuela	2	15	67
	Malá	3	Moncholo	2	Charata	12	Du Graty	3		
					Gancedo	2	Saenz Peña	1		
					Las Breñas	15				
					Machagai	4				
					Las Chuñas	1				
					Saenz Peña	7				
					Tres Isletas	3				
Eastern	Barranqueras	2	Argüello	12	Basail	10	Antequeras	1	20	124
	Negro	11	Colussi	10	Escondida	6	Resistencia	1		
	Paraná	12	Los Lirios	27	La Leonesa	1				
	Tragadero	2	Palma	4	Las Palmas	1				
			Prosperidad	3	Makallé	1				
			Tigre	6	Pto. Tirol	4				
					Resistencia	5				
					San Martín	2				
Total	6	33	8	67	16	83	5	8	35	191

Water samples were collected in sterile 250-ml polypropylene bottles, according to internationally recommended methodology (APHA, 1999; WHO, 2004). Samples were kept at 4°C until their arrival to laboratory.

2.3. Detection and identification of *Enterobacteriaceae*

The presence-absence coliform test was used as method for enrichment. Liquid media showing bacterial growth were streaked onto Eosin Methylene Blue Agar and incubated at 35°C during 24 hours for enterobacteria recovery (APHA, 1999; WHO, 2004). Isolates were identified by classic biochemical tests (Schreckenberger et al., 1999).

2.4. Antimicrobial susceptibility testing

The antimicrobial resistances were evaluated by the agar disk diffusion method (CLSI, 2005). Antibiotics selected for testing were those more frequently used in human medical practice (Famiglietti et al., 2005).

The protocol for *Escherichia coli* susceptibility testing included the following antimicrobial agents: ampicillin 10 µg, ampicillin+sulbactam 10 µg/10 µg, cephalothin 30 µg, cefoxitin 30 µg, cefotaxime 30 µg, ceftazidime 30 µg, gentamicin 10 µg, chloramphenicol 30 µg, sulfamethoxazole-thrimethoprim 23.75 µg/1.25 µg, nalidixic acid 30 µg, and ciprofloxacin 5 µg.

Antimicrobials tested for non-*E. coli* isolates were: nalidixic acid 30 µg, chloramphenicol 30 µg, gentamicin 10 µg, sulfamethoxazole-thrimethoprim 23.75 µg/1.25 µg, ciprofloxacin 5 µg, cefotaxime 30 µg, and ceftazidime 30 µg.

Escherichia coli ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212 were used as control in antimicrobial susceptibility tests.

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2.5. Statistical analysis

Chi-Square was applied to determine the association between different water sources and antimicrobial resistance (at 5% significance level).

3. RESULTS

3.1. Detection and identification of *Enterobacteriaceae*

A total of 191 *Enterobacteriaceae* isolates were obtained from 506 samples analyzed. The distribution of species according to the source of water is presented in Table 2. *Escherichia* and *Klebsiella* were the most frequent genera recovered from all sources. There were not significant difference between the isolates obtained from surface water and ground water for *Escherichia coli* ($p = 0.81$), *Klebsiella* spp ($p = 0.11$), *Enterobacter* spp ($p = 0.51$), and *Citrobacter* spp ($p = 0.78$). Regarding to other enterobacteria, the difference was statistically significant in favor of those isolated from ground water ($p = 0.049$), although their number was considerably smaller in comparison with the genera above mentioned.

Table 2. Number (n) and percentage (%) of *Enterobacteriaceae* isolates obtained from water samples in NE Argentina, according to the source.

Source	<i>E. coli</i>		<i>Klebsiella</i> spp.		<i>Enterobacter</i> spp.		<i>Citrobacter</i> spp.		Others		Total	
	n	%	n	%	n	%	n	%	n	%	n	%
Surface water	36	52.9	35	62.5	14	45.2	14	56.0	3	27.3	102	53.4
Ground water	30	44.1	19	33.9	14	45.2	10	40.0	8	72.7	81	42.4
Drinking water	2	2.9	2	3.6	2	6.5	1	4.0	0	0.0	7	3.7
Total	68	100.0	56	100.0	31	100.0	25	100.0	11	100.0	191	100.0

3.2. Antimicrobial susceptibility testing

Antibiotic resistance found in *Escherichia coli* isolates according to the origin of water samples is shown in Table 3.

Table 3. Antibiotic resistance of *Escherichia coli* isolates recovered from water samples in NE Argentina, according the origin.

Antibiotics	Source			Total (n=68)
	Surface Water (n=36)	Ground Water (n=30)	Drinking water (n=2)	
Ampicillin	17 (47.2%)	12 (40.0%)	1 (50.0%)	30 (44.1%)
Ampicillin-sulbactam	4 (11.1%)	0 (0.0%)	0 (0.0%)	4 (5.9%)
Cephalothin	12 (33.3%)	6 (20.0%)	0 (0.0%)	18 (26.5%)
Sulfamethoxazole-thrimethoprim	4 (11.1%)	0 (0.0%)	0 (0.0%)	4 (5.9%)
Chloramphenicol	3 (8.3%)	0 (0.0%)	0 (0.0%)	3 (4.4%)
Nalidixic Acid	3 (8.3%)	0 (0.0%)	0 (0.0%)	3 (4.4%)

Among 30 ampicillin-resistant *Escherichia coli* isolates, 4 were also resistant to ampicillin/sulbactam. There were no significant differences ($p = 0.5$) between the antimicrobial resistance of the surface water isolates and the ground water isolates.

Among the genus *Klebsiella*, 41 *K. pneumoniae* strains, 14 *K. oxytoca* and 1 *K. planticola* were identified; only one *K. pneumoniae* strain from surface water was resistant to nalidixic acid and chloramphenicol.

Twenty isolates of *Citrobacter* sp were obtained; 5 isolates of *C. freundii* recovered from ground water were resistant to nalidixic acid but sensible to the rest of the assessed antibiotics.

In relation to the genus *Enterobacter*, 30 strains were recovered: 4 *E. cloacae*, 6 *E. aerogenes*, 6 *E. gergoviae*, and 14 *Enterobacter* spp. In this genus, only 1 isolate from ground water was resistant to the nalidixic acid.

In the present work was not found any extended-spectrum- β -lactamase-producing nor ciprofloxacin-resistant strain, nevertheless, those nalidixic acid-resistant isolates must be considered as strains with decreased susceptibility for ciprofloxacin (CLSI, 2005).

4. DISCUSSION

High frequencies of antimicrobial resistance have been reported in bacteria recovered from environmental samples (Boon et al., 1999), and because the antibiotic resistance may be spread from resistant to formerly sensitive strains, the maintenance of antibiotic resistance in environmental isolates may act as a reservoir for antibiotic-resistance encoding genes.

Studies conducted in various countries have detected a number of antibiotics in the low microgram per liter or the nanogram per liter range in different environmental compartments (Kümmerer, 2004).

Antibiotics are present in the environment at sub-inhibitory concentrations as a consequence of hospital effluents, municipal sewage and wastewater from livestock farms or agricultural use, and its presence allows the selection of resistant strains. The presence of several antibiotic compounds in both sewage treatment plants effluents and surface waters result most likely from applications in human medicine than from veterinary uses (Hirsch et al., 1999). Added to this fact, bacteria with acquired resistance have been also found in surface water, ground water, and drinking water in different countries (McKeon et al., 1995; Schwartz et al., 2003; Córdoba et al., 2001).

Data obtained in this work about frequencies of resistant enterobacteria in water sources are in agreement with those carried out in Brazil by Lourenço et al. (2007), in United States by McKeon et al. (1995), and in South Africa by Lin et al. (2004), where *E. coli* was the most frequent specie followed by *Citrobacter*, *Enterobacter* and *Klebsiella*.

The high rate of resistance to ampicillin found in *E. coli* (44.1%) in this work is superior to those previously reported in Argentina (Baldini et al., 1991) and in Australian in estuarine and river waters (Boon et al., 1999), but they are lower than those reported by Alhaj et al. (2007) in Malaysia and by Junco-Díaz et al. (2006) in Cuba. Nevertheless, our results are in agreement with those found in rivers that collect agricultural and industrial drainages in South Africa (Lin et al., 2004). These discrepancies may be due to the sources of contamination, since an important difference was found when contaminant bacteria came from domestic, wild-animals or human sewage (Sayah et al., 2005).

In this work, 26 *Escherichia coli* isolates presented a phenotype consistent with the presence of a plasmidic-broad-spectrum- β -lactamase (TEM-like), which hydrolyse ampicillin but is inhibited by sulbactam. This number is higher than the 4.9% found in *Escherichia coli* from recreational waters in Canada, which harboured the *bla_{TEM}* gen that codifies for a plasmidic lactamase (Hamelin et al., 2006).

Only 4 *Escherichia coli* strains showed a phenotype consistent with a chromosomal hyperproduced β -lactamase which is non-inhibited by sulbactam (AmpC-like) (Risueño et al., 2002). Although some authors detected the presence of *AmpC* genes in wastewater, surface water, and drinking water, this class C Ambler's β -lactamase is naturally harbored by a number of bacteria such as *Escherichia coli* and *Shigella*, but in wild state it is expressed at very low level and does not confer resistance to the aminopenicillins, except in those AmpC-hyperproducing strains (Livermore, 1995).

In this work there were not significant differences between the resistance levels to ampicillin among *Escherichia coli* isolates with those previously published by Whonet in clinical strains obtained from outpatients in Argentina (PAHO, 2005). This finding would indicate that in aquatic environments there have been a selection of resistant strains or that the strains were introduced in the water from human or animal sources.

The rate of resistance to nalidixic acid found by Goñi-Urriza et al. (2000) in *Enterobacteriaceae* isolated from urban effluents is close to the rate found in this work, but their findings on resistance to chloramphenicol is higher than ours.

E. coli isolates in this work presented low levels of antimicrobial resistance, which is in agreement with Lin et al. (2004), although they differ with other reports about high levels of antimicrobial-resistant bacteria isolated from rural groundwater supplies (McKeon et al., 1995) or from rivers, reservoirs, and sewage treatment facilities (Boon et al., 1999).

Even when only one ampicillin-resistant *Escherichia coli* strain was isolated from drinking water in our work, other authors from Germany (Schwartz et al., 2003) and from Argentina (Córdoba et al., 2001) have found many strains resistant to several antibiotics in the same kind of samples.

Some studies carried out on a large-scale distribution pilot system showed that *E. coli* can survive for several days in a dead-end section of the system, but does not multiply within the pipeline (Leclerc, 2003). This could be indicating that antimicrobial-resistant *E. coli* can reach the distribution systems by contamination of the cisterns or through leakage in the pipelines, and that they are not killed by residual chlorine concentrations. Nevertheless, the role of the environment in the emergence and spreading of antibiotic resistance bacteria, its possible pathways, and the way in which environmental bacteria contribute to the spread of resistance genes are not clearly stated so far (Schwartz et al., 2003).

Antimicrobials may have irreversible effects in the environment, even at very low concentrations, situation that is not caused by other chemical compounds. Additionally, the 5-10% of bacteria present in water is viable but non-culturable and that is why the study of antibiotic resistance allows evaluating a small fraction of the impact of antibiotics in aquatic ecosystems (Séveno et al., 2002).

There is clear evidence that if resistant bacteria enter in local waterways, their presence is associated with the risk of transferring resistant genes from harmless bacteria to pathogenic bacteria or to humans interacting with aquatic environments (Costanzo et al., 2005). Thus, water quality managers need to identify the source/s of pollution in watersheds and generally they are primary interested in discriminating if contamination is of animal or human origin, and secondarily in determining the major source of animal contamination (Harwood et al., 2000).

5. CONCLUSIONS

The rates of antibiotic resistance found in *Enterobacteriaceae* strains recovered from environmental sources were higher than expected for wild strains. Resistance encountered can be interpreted as an indicator of the extended use of antibiotics in the region and/or the contamination of water sources with resistant bacteria originated in hospital environments. The findings of the present work are indicating that the occurrence of antimicrobial-resistant bacteria in aquatic ecosystems in the Province of Chaco may contribute significantly to environmental antibiotic resistance dissemination.

6. ACKNOWLEDGEMENT

This work was supported by Fundación “Alberto J. Roemmers”.

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