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Antibiotic Resistance in Seawater Samples From the Spanish East Coast

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Abstract: Seawater has been proposed as a reservoir for antibiotic resistant bacteria and antibiotic resistance genes, thus representing a risk to public health. In this study, we evaluated the presence of antibiotic resistance determinants (bacteria and genes) in 77 seawater samples collected at different points along the coast of the Gulf of Valencia (Spain). Specifically, indicators of fecal contamination bacteria, *Escherichia coli* and *Enterococcus* sp. were isolated, and their antibiotic resistance profiles were analyzed by using the Sensititre® system, followed by the detection of main antibiotic resistance genes (*bla*TEM, *qnr*S, *tet*W, *sul*I, and *erm*B). The highest frequencies of resistance in *E. coli* isolates were detected against ampicillin (35.1%) and ciprofloxacin (17.5%), followed by sulfamethoxazole and trimethoprim (15.7%), while 23% of enterococci isolates showed resistance to a single antibiotic, 20% against tetracycline and 3% against daptomycin. By PCR, 93% of the *E. coli* strains showed the *bla*TEM and *sul*I resistance genes. Among the enterococci, the presence of the *bla*TEM gene was detected in 40% of the isolates, while the rest of the genes were present at very low rates. Among the water samples, 57% were positive for at least one of the tested genes, being *bla*TEM gene the most commonly found (47%), followed by *qnr*S (33%) and *sul*I (23%) genes. These results show that seawater, in addition to having a high rate of fecal contamination, can contribute to the spread of antibiotic resistance.

Keywords: antibiotic resistance; fecal indicators; seawater; Mediterranean

1. Introduction

Antimicrobial resistance (AMR) is one of the world's greatest public health challenges [1,2]. The spread of antibiotic-resistant bacteria (ARB) is directly linked to the overuse and inappropriate use of antibiotics in human and veterinary medicine, aquaculture, and agriculture, as well as inadequate disposal in several regions of the world [3]. Although most studies have focused on resistance in clinical settings, the increase in infections caused by ARB has stimulated interest in understanding the occurrence of antibiotic resistance in natural settings. The environment plays a crucial role as a potential reservoir of antibiotic resistance genes (ARG) [4,5] contributing to their spread among environmental bacteria and human pathogens via horizontal gene transfer (conjugative plasmids and integrons) [6-8].

AMR is one of the central points of the "One Health" approach and any strategy aimed at reducing the risk to human health due to AMR must consider, not only the health context, but also all the complex interrelationships that occur between all the ecological niches that act as reservoirs and sources of dispersion of these resistance determinants [9,10]. Several studies reported the presence of antibiotic-resistant bacteria and/or resistance genes in different environments such as soils [11], vegetables [12,13], livestock farms [14], and aquatic environments such as wastewater [15,16], drinking water [17-20], surface waters [21-23], irrigation ditches [24,25] and to a lesser extent the marine environments. However, it is well known that run-off from land-based sources and wastewater discharges can reach the sea introducing resistant bacteria and their genes [26]. Another important source of antimicrobial resistance is the use of antibiotics in activities related to marine aquaculture [27,28]. Therefore, seawater can diffuse ARG not only through the fluidity of water

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bodies but also through transfer between bacterial species [29]. Furthermore, urban coastal beaches, which are widely used by humans for recreational purposes, may provide an ideal environment for resistance spread [30,31].

Although ARB and ARG have been recognized as emerging contaminants [32,33], insufficient information is available on their prevalence and abundance in the environment [34]. Therefore, information on the environmental occurrence of ARB and ARG will help to fill existing gaps and support national and international action plans to reduce the spread of antimicrobial resistance [35].

Although, as mentioned above, ARG could be transferred to the sea, reports on ARG pollution in the coastal areas are very few. Hence, this study aims to evaluate the antibiotic resistance profile of fecal indicator bacteria isolated from seawater from different coastal locations in Comunitat Valenciana (Spain) by combining phenotypic and molecular analyses. ARG that confer resistance to the most used antibiotics were selected: β-lactams (*bla*TEM), macrolides (*erm*B), fluoroquinolones (*qnr*S), sulfonamides (*sul*I), and tetracyclines (*tet*W). These genes have been shown to be significant in estuarine and marine environments [36].

2. Materials and Methods

2.1. Samples

A total of 77 seawater samples were taken along 23 coastal locations of the Comunitat Valenciana (East of Spain). Twenty-one seawater samples were collected from eight municipalities of North Valencia province, twenty-three samples were collected from nine municipalities of South Valencia province and twenty-six were from six municipalities of Alicante province. Figure 1 shows the sampling locations along the coast.

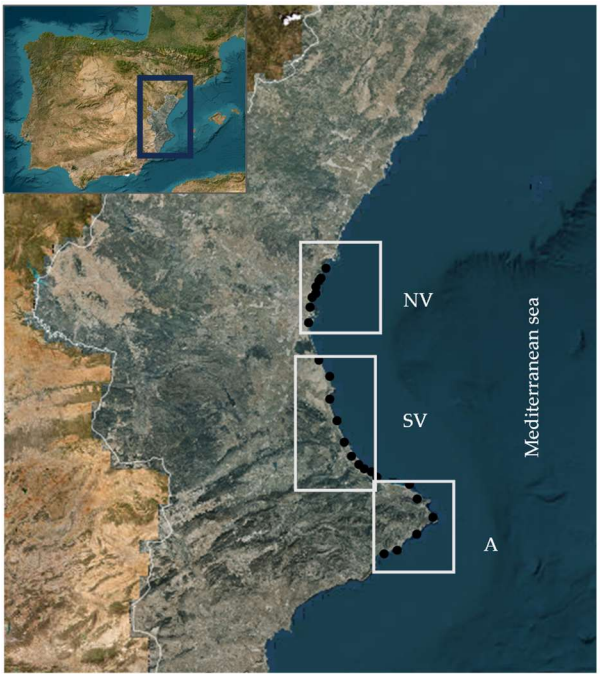

Figure 1. Coastal water sampling sites.

All samples were collected aseptically, placed in a temperature-controlled box at 4 ºC, transported to the laboratory, and processed without further delay.

2.2. Bacterial analysis

Bacterial analyses were performed using Colilert and Enterolert Test Kits to detect *Escherichia coli* and *Enterococcus* sp., respectively, according to manufacturer instructions. Both methods have been certified as alternative methods by the standards ISO 9308-2 and ISO 7899-1. The density units

are expressed as the most probable number (MPN) per 100 mL, according to Quantry-Tray/2000 and Quanty-Tray systems, respectively (IDEXX Laboratories). *E. coli* and *Enterococcus* sp. colonies were isolated from positive wells onto Tryptone Bile X-Glucuronide (TBX) Agar (Oxoid, Ltd., Hampshire, UK) and Bile Esculine Azide Agar (Scharlau, Barcelona, Spain) respectively, and stored in refrigeration until their use.

2.3. Determination of antibiotic resistance

Antibiotic resistance of *E. coli* and *Enterococcus* sp. isolates was determined using the Sensititre System (Thermo Scientific), an automatic system that uses a microplate format with a panel of different antimicrobial compounds that are accurately dosed at appropriate dilutions resembling the classical broth dilution method, according to the methods described by the Clinical and Laboratory Standards Institute [37], and accepted as method of international reference (ISO 20776-1: 2006). The test was performed following the manufacturer's instructions. Results are read by means of a digital visualization system Sensititre Vizion (Thermo Scientific); for those wells that show growth, a sedimentation button is observed, indicating the resistance of the microorganism to the concentration of antibiotic contained in the well. The well containing the lowest antimicrobial concentration where no growth is observed is the MIC. *E. coli* ATCC 25922 and *E. fecalis* ATCC 29212 were used as internal controls.

2.4. Detection of ARG

Gene detection has been carried out both from the direct water samples and bacterial isolates using GenElute ™ Bacterial Genomic DNA Kit (Sigma-Aldrich. ref. NA2110). For water samples, a volume of 250 mL was filtered through a 0.45 µm pore-size membrane (Millipore) to retain bacterial cells. The membranes were aseptically cut and suspended in 500 µL of lysis buffer included in the kit; 80 mg of glass beads were added, and the mixture was shaken for 20 min at 3000 rpm (Disruptor Gene (USA Scientific). Genomic DNA was extracted in accordance with the manufacturer's instructions.

For ARG detection from *E. coli* and enterococci isolates, bacterial cells from an overnight culture were harvested, suspended in 500 µL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8), and then, DNA was extracted following the manufacturer's instructions.

The ARG chosen for this study were *bla*TEM, *erm*B, *qnr*S, *sul*I and *tet*W, which confer resistance to β-lactam antibiotics, macrolides, quinolones, sulfonamides, and tetracyclines, respectively.

The PCR mix was prepared in a total volume of 50 µL containing 5 µL DNA template, and 45 µL of reaction mixture including 1X PCR buffer (Sigma-Aldrich), 1.5 mM $MgCl_2$ (Sigma, Aldrich), 0.2 mM dNTPs, 0.25 µM of each primer (TIB MOLBIOL) and 2.5 U Taq polymerase (Sigma, Aldrich). The primer sequences and cycling conditions for five PCR are shown in Table 1. MiliQ water is used as a negative control and *E. coli* isolates positive for each investigated gene, were used as positive controls. The PCR products were analized by gel gel electrophoresis on 1.2% agarose gel in 1xTAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) plus 5 µL RedSAfe (iNtRON Biotechnology) per 100 mL. Ten µL of PCR product, previously mixed with 6X loading buffer, was used. Two molecular weight markers are included in each gel (GeneRuler 100 bp DNA Ladder, 0.5 µg/µL, ThermoFischer). Finally, the fragments were visualized in a transilluminator under UV light.

Table 1. PCR primer sequences, targets, and conditions of reactions.

Target gene	Sequence	Conditions	Reference
*bla*TEM	5′ -GCKGCCAACTTACTTCTGACAACG- 3′ 5′ -CTTTATCCGCCTCCATCCAGTCTA- 3′	95ºC 3 min (1 cycle); 95ºC 15 s and 60ºC 20 s (40 cycles); 72 ºC 1 min	[38]
*erm*B	5′ -GATACCGTTTACGAAATTGG- 3′ 5′ -GAATCGAGACTTGAGTGTGC- 3′	95ºC 3 min (1 cycle); 95ºC 15 s and 58ºC 20 s (40 cycles); 72 ºC 1 min	[39]

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*qnr*S	5′ -GACGTGCTAACTTGCGTGAT- 3′ 5′ -TGGCATTGTTGGAAACTTG- 3′	95ºC 3 min (1 cycle); 95ºC 15 s and 62ºC 20 s (40 cycles); 72 ºC 1 min	[40]
*sul*I	5′ -CGCACCGGAAACATCGCTGCAC- 3′ 5′ -TGAAGTTCCGCCGCAAGGCTCG- 3′	95ºC 3 min (1 cycle); 95ºC 15 s and 65ºC 20 s (40 cycles); 72 ºC 1 min	[41]
*tet*W	5′ -GAGAGCCTGCTATATGCCAGC- 3′ 5′ -CTTTATCCGCCTCCATCCAGTCTA- 3′	95ºC 3 min (1 cycle); 95ºC 15 s and 60ºC 20 s (40 cycles); 72 ºC 1 min	[42]

3. Results

3.1. Microbiological determination of E. coli and Enterococcus sp. in marine water samples

Among the 77 water samples analyzed, 74% showed contamination by *E. coli* and 39% by *Enterococcus* sp. The NMP of CFU/100 mL varied from 1 to 3.8 $\log_{10}$ and 1 to 2.9 $\log_{10}$ for *E. coli* and *Enterococcus* sp., respectively. Statistical analysis did not show significant differences in the contamination level among the three sampling zones. In accordance with the limits established in Spanish regulations (R.D. 1341/2007), 87% of the analyzed waters presented excellent quality (87%) as bathing waters while the rest were classified in the good quality category.

3.2. Antibiotic resistance determination of bacterial isolates

A total of 87 strains were isolated from seawater samples (57 *E. coli*, and 30 *Enterococcus* sp.) and subjected to antibiotic sensitivity test. The resistance of the bacteria to antibiotics was determined according to the CLSI standards [37] for antimicrobial susceptibility testing.

Among the 57 isolates of *E. coli* (Table 2), 34 isolates (59.6%) were resistant to at least one antibiotic. Resistance to ampicillin was the most frequent (20 isolates) followed by ciprofloxacin (10 isolates), sulfamethoxazole, and trimethoprim with 9 isolates for each one, and tetracycline and nalidixic acid with 8 isolates. MIC values for these antibiotics were 32 - > 64 mg/L, 0.125 - 0.5 mg/L, > 1024 mg/L, > 32 mg/L, 32 - ≥ 64 mg/L, and >128 mg/L, respectively. There was no correlation (p > 0.05) between the number of strains resistant to any antibiotic and the area from which they were isolated. Multiple resistance (≥ 3 antibiotic classes) [43] was observed in 5 out of 34 resistant *E. coli*, (14.7%). Each of them presented a unique multi-resistance pattern. β-lactamic resistance was present in all profiles, followed by sulfonamide- and tetracycline-resistance, present in 4 profiles, and quinolone resistance, observed in 3 profiles. Resistance to polymyxins (colistin) and aminoglycosides (gentamicin) was only present in one multi-resistance profile each. Finally, none of the isolates showed resistance against chloramphenicol and tigecycline, with MIC range of ≤ 8-16 mg/L and ≤ 0.25 - 1 mg/L, respectively.

Only 7 *Enterococcus* sp. isolates showed resistance to the tested antibiotics, specifically, 6 isolates against tetracycline and 1 against daptomycin. MIC range for these antibiotics was 64 -128 mg/L, and 16 mg/L, respectively. Statistical analysis showed no correlation (p > 0.05) between the resistance of isolates to any antibiotic and the area from which they were isolated.

Table 2. Incidence of resistance for each antimicrobial compound separately and of multi-resistance in 3 different zones of Comunitat Valenciana Coast.

Zone	No. tested isolates	Number of *E. coli* resistant isolates													
		SMX	TMP	CIP	TET	MER	AZI	NAL	FOT	CHL	TGC	TAZ	COL	AMP	GEN	MDR[a]
NV	14	2	3	2	2	0	1	3	0	0	0	1	0	4	1	2
SV	23	4	4	2	2	1	3	4	2	0	0	4	1	12	0	2
A	20	3	2	4	4	0	1	1	0	0	0	0	0	4	0	1
Total	57	9	9	10	8	1	5	8	2	0	0	5	1	20	1	5

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% of resistant isolates	15.8	15.8	17.5	10.4	1.8	8.8	14	3.5	0	0	8.8	1.8	35.1	1.8	14.7

SMX: sulfamethoxazole; TMP: trimethoprim; CIP: ciprofloxacin; TET: tetracycline; MER: meropenem; AZI: Azithromycin; NAL: nalidixic acid; FOT: cefotaxime; CHL: chloramphenicol; TGC: tigecycline; TAZ: ceftazidime; COL: colistin; AMP: ampicillin; GEN: gentamicin. NV: North Valencia; SV: South Valencia; A: Alicante. [a]: MDR: resistance to ≥ 3 antibiotic classes.

3.3. Antibiotic resistant gene determination in water samples and bacterial isolates

The presence of five antibiotic-resistance genes in the 77 seawater samples was analyzed as described in the Material and methods section. Forty-four of them (57%) were positive for at least one of the genes. As shown in Table 3, *bla*TEM gene was the most frequently detected (46.7%), followed by *qnr*S (32.5%) and *sul*I (23.4%); *tet*W gene was detected in 17% of the samples, while *erm*B was only present in one sample. The statistical study showed only a significant relationship between the *tet*W gene and the area of Alicante (χ^2=10.384, $p < 0.05$), and it was not detected in any of the samples from South Valencia. Combinations of two genes were detected in 24 seawater samples (31%), being the most abundant combinations of those containing *bla*TEM gen; three gene combinations were detected in 9 samples (ca. 12%). Finally, four and five gene combinations were present in only one sample. A statistically significant dependence relationship was detected only between *tet*W and the sampling zone (χ^2 = 10,384, $p < 0,05$) as this gene was not detected in any sample of South Valencia.

Table 3. Presence of antibiotic resistance genes in water samples according to sampling zone.

Zone	No. samples	Number of positive samples				
		***bla*TEM**	***qnr*S**	***erm*B**	***sul*I**	***tet*W**
NV	21	6	5	0	3	5
SV	30	15	9	0	5	0
A	26	15	11	1	10	8
TOTAL (%)	77	36 (46.7)	25 (32.5)	1 (1.2)	18 (23.4)	13 (16.9)

NV: North Valencia; SV: South Valencia; A: Alicante.

The *bla*TEM together with the *sul*I genes, were the most frequently detected, each of them being found in 53 *E. coli* strains (92.9%) (Table 4). They were followed in frequency by the *tet*W and *qnr*S genes, present in 40% and 36.8% of the samples, respectively. In contrast, the *erm*B gene was detected in only 3 isolates (5%). Statistical analysis showed no dependence relationship between the sampling area and the frequency of gene detection in *E. coli* isolates ($p > 0.05$).

Table 4. Presence of antibiotic resistance genes in E. coli isolates according to sampling zone.

Zone	No. samples	Number of positive samples				
		***bla*TEM**	***qnr*S**	***erm*B**	***sul*I**	***tet*W**
NV	14	13	4	0	13	2
SV	23	21	11	1	21	11
A	20	19	6	2	19	10
TOTAL (%)	57	53 (92.9)	21 (36.8)	3 (5.2)	53 (92.9)	23 (45.1)

NV: North Valencia; SV: South Valencia; A: Alicante.

The analysis of genes in enterococci again showed that the blaTEM gene was the most abundant, detected in 40% of the isolates. The percentages of the other genes ranged from 3 to 7%. Statistical

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analysis showed no relationship between the sampling area and the occurrence of genes in the isolates ($p > 0.05$).

For most of the genes investigated, their presence was greater in the isolates than in the directly analyzed waters (Figure 2). In North Valencia, the blaTEM and sulI genes were detected at significantly higher levels in the E. coli isolates than in the seawater samples ($\chi^2 = 13.988$, $p = 0.0002$ and $\chi^2 = 20.896$, $p = 0.000$, respectively). In South Valencia, besides the blaTEM and sulI genes ($\chi^2 = 10.194$, $p = 0.0014$ and $\chi^2 = 29.020$, $p = 0.000$, respectively), significant differences were found in the tetW gene, which was not detected in any of the seawater samples ($\chi^2 = 18.106$, $p = 0.0000$). In Alicante, significant differences were found in the blaTEM and sulI genes, which were detected to a greater extent in the isolates ($\chi^2 = 8.160$, $p = 0.0043$ and $\chi^2 = 15.120$, $p = 0.0001$, respectively).

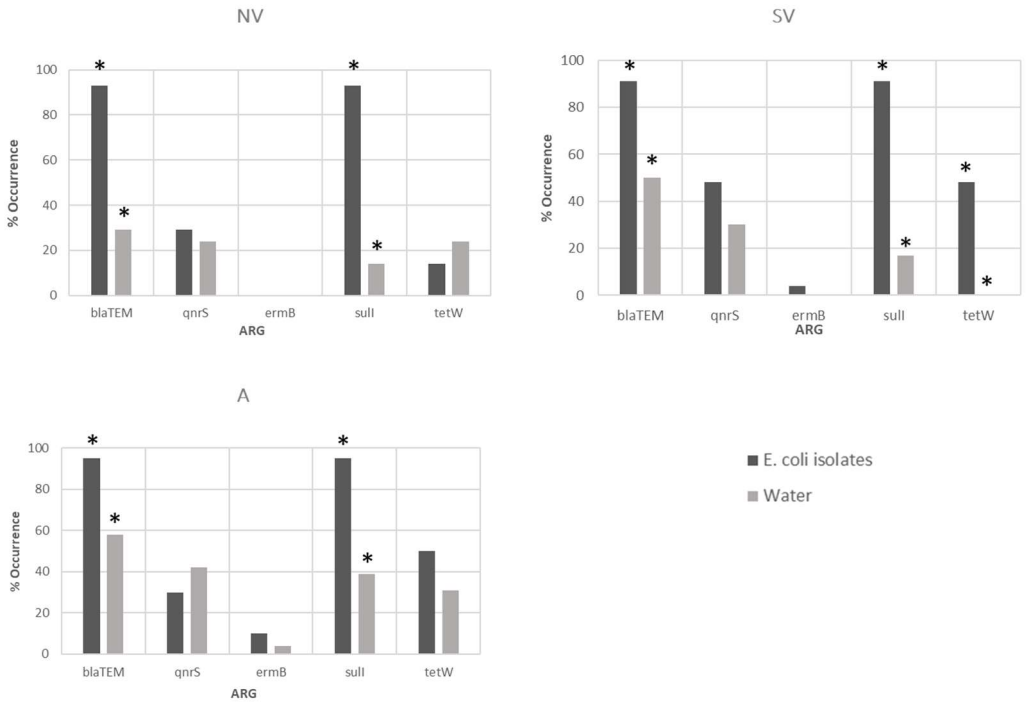

Figure 2. Comparison of ARG occurrence among the isolates and water of the different emplacements (NV: North Valencia; SV: South Valencia; A: Alicante). * : Significant differences ($p < 0.05$) between gene occurrence in water samples and in E. coli isolates.

4. Discussion

In recent decades, humanity has been confronted with the growing problem of the spread of antibiotic-resistant bacteria (ARB) and the difficulty of treatment of associated infections. The presence of these bacteria in coastal environments, as well as the presence of ARG circulating freely in the water, represents a serious problem, especially for people who visit these areas for leisure [44] owever, data on their presence and importance in the marine environment are still limited.

Thus, 77 water samples from the coastal waters of Comunitat Valencia (Eastern Spain) were analyzed using microbial and molecular methods, to determine the presence of ARB and ARG. The main species studied included E. coli and Enterococcus sp. which were tested for resistance to 12-14 antibiotics. The detected levels of both microorganisms are within the range found in the compilation of several studies in marine waters worldwide embracing four decades of research [45], ranging from 0.59 to 3.51 $\log_{10}$ CFU/100 mL for E. coli and from 0.63 to 3.21 $\log_{10}$ CFU/100 mL for Enterococcus sp. In a macro study conducted in Spain between 2012 and 2015, covering 1392 beaches throughout the country, a mean of 1.4 $\log_{10}$ CFU/100 mL for E. coli and 1.32 $\log_{10}$ CFU/100 mL for Enterococcus sp. was found for the southern Mediterranean, where our samples were collected, with a national mean of 1.93 and 1.65 $\log_{10}$ CFU/100 mL for each of the microorganisms [46]. Our values were within these averages, although we found some samples with E. coli values of 4.4 $\log_{10}$ CFU/100 mL and some

samples of Enterococcus sp. of 4.2 $\log_{10}$ CFU/100 mL; both results are above acceptable levels and could be due to the spillage of fecal matter due to a problem with the sewerage system or run-off from rainfall. It should also be noted that these exceptionally polluted samples were taken in summer, on beaches close to the city of Valencia, where not only part of the local population (Valencia and its metropolitan area have a population of around one and a half million inhabitants) but also many tourists bathe during these periods. Some authors have already stated that beaches in urban environments, as in our case, have a lower quality than those in semi-urban or natural environments [47-50], which is related to the number of people using the beaches, as well as the pets and birds, especially seagulls, that have access to them [51-53]. It is, therefore, difficult to determine the origin of this contamination.

The search of ARB was directed to the detection of E. coli and enterococci of fecal origin. Both microorganisms have been recognized as important actors in the spread of antibiotic resistance [54,55]. The percentage of E. coli strains resistant to at least one antibiotic (59.6%) was similar to that obtained in a study conducted on beaches in Brazil [56], and lower than other studies in Portugal [54] or China [57], with percentages of up to 80%. Ampicillin resistance was the most frequent, confirming that β-lactam resistance is the most prevalent in the aquatic environment [58,59]. Other antibiotics, such as ciprofloxacin, nalidixic acid, tetracycline, and sulfamethoxazole, had resistance rates ranging from 14% to 18%. Similar findings were made by Hernández et al. [60] in a study conducted in Antarctica, an extreme location where the human presence is due to the establishment of scientific bases and where the removal of fecal contamination is very strictly controlled. This reflects the general resistance to β-lactams worldwide, with varying rates of resistance to other antibiotics depending on various factors such as geographical location. This fact also demonstrates the ability of antimicrobial resistances to distribute homogeneously in seawater.

Nine percent of the E. coli strains showed multidrug resistance, and again, the β-lactam family was present in all the resistance profiles. Comparing these data with other studies, we see very different percentages of multidrug resistance, supporting the idea that the majority of multidrug-resistant ARB are not of environmental origin, but rather originate in places of high selective pressure, such as hospitals or highly contaminated ecosystems. The levels found in each geographical area are very consistent with the antibiotic use policies in these areas [61].

Enterococci showed low levels of resistance (23%) compared to other studies conducted on Mediterranean beaches, for example, in Italy, with 38% of resistant strains [62] or in Tunisia, where this resistance is as high as 75% [63]. In our study, only strains resistant to tetracycline (20%) and daptomycin (3%) were obtained, the latter being a rarely used drug. However, it is important to note that daptomycin is used as a last line of therapy, especially in vancomycin-resistant enterococcal infections [64]; therefore, the emergence of daptomycin-resistant enterococci is of concern in healthcare settings. No multidrug-resistant Enterococcus spp. isolates were found. These data are encouraging, as antibiotic-resistant enterococci found in coastal marine waters are often closely related to human clinical isolates, probably originating from urban wastewater [65,66].

PCR assays revealed that blaTEM was the most frequently detected gene in the analyzed seawater samples. These results are in line with other studies in the Mediterranean, where this gene was detected in almost all samples [54,67], confirming that β-lactam antibiotics resistance genes are widely distributed in seawater samples. The presence of the qnrS , sulI and tetW genes is also comparable with the results of some other works that attest to their prevalence in coastal environments [68], but it contrasts with the absence detected in other coastal waters, such as Sicily [67].

Once again, the blaTEM gene was the most frequently detected gene in both E. coli (92.9%) and Enterococcus sp. isolates (40%). The remaining genes showed more variable percentages, with the exception of sulI in E. coli isolates, which also stands out with identical percentages to blaTEM Other studies demonstrated that blaTEM, tet, and sul genes are ubiquitous in E. coli isolates from estuarine and marine environments [69-72].

ARG were detected to a greater extent in E. coli isolates than in direct water samples. These results contrast with those found in other types of water, such as the study carried out by Amato et

al. [73] in ditch water flowing into the sea, specifically in the North Valencia zone. This could be due to the fact that the marine environment is a diluting medium, so the filtrate could provide little genetic material for amplification, whereas for the detection of E. coli ARG, pure colonies with a high DNA load are used as a starting point.

Antibiotic resistance is no longer just a clinical problem [74]. The demonstration of the presence of antibiotic resistance (bacteria and genes) in this work highlights the role of the sea in the spread of AR genes and the need to carefully consider the risks to human health associated with fecal contamination. Coastal areas, where human recreation, in addition to commercial activities, are important, are ideal environments for the transmission of resistance to the human population.

5. Conclusions

This study provides a clearer perspective on the circulation of ARG in the marine environment and its relationship with fecal pollution in seawater. This could facilitate future interventions to reduce antimicrobial resistance in the environment as part of a holistic One Health approach.

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