

## STUDY ON ANTIBIOTIC RESISTANCE IN POTENTIAL PATHOGENIC BACTERIA ISOLATED FROM NHA TRANG BAY, VIETNAM

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### SUMMARY

Antimicrobial resistance (AMR) is recognized as a critical threat to global public health. However, research efforts have predominantly focused on terrestrial systems, with marine habitats receiving comparatively less attention. This study investigates the prevalence and distribution of drug-resistant bacteria in the marine environment of Nha Trang Bay, Vietnam. 53/106 (50%) bacterial species isolated from surface water and sediment samples were identified as potential pathogens to human and animal health. The most prevalent pathogens included *Pseudomonas aeruginosa* (20.6%), *Klebsiella pneumoniae* (15.1%), *Escherichia coli* (13.2%) and *Citrobacter freundii* (7.5%). Antibiotic susceptibility testing revealed that 60.4% (32/53) pathogens were resistant to at least one antibiotic tested, in which 28.3% (15/53) isolates exhibited multidrug-resistant phenotypes. The resistance proportion was highest for Amoxicillin/clavulanic acid (80%), followed by ticarcillin, cefoxitin, nalidixic acid, and cefotaxime. Finally, 9 antibiotics-resistant genes including *sul1*, *sul2*, *tetA*, *tetQ*, *tetB*, *blaKPC*, *blaVIM*, *vanA* and *mecA* were detected. These findings highlight Nha Trang Bay as a significant reservoir for emergence and transmission of drug-resistant bacteria in coastal areas, posing substantial risks to food safety, human and animal health.

**Keywords:** Multidrug resistance, antibiotic-resistant genes, pathogenic bacteria, marine environment, Nha Trang Bay.

### INTRODUCTION

Antibiotic-resistant infections currently kill approximately 700,000 people per year around the world and are predicted to cause 10 million deaths per year by 2050. Bacteria represent the pathogens the most often (54,3%) involved in emerging infectious disease events in the last decades, and each year antibiotic-resistant bacteria (ARB) accounted for an estimated 68,000 deaths in the European Union and the United States, along with the economic losses of 55 billion (Avershina *et al.*, 2021). In many regions in the world, particularly in low and middle-income countries, the emergence of multidrug-resistant strains is highly concerning. Many bacterial strains even were resistant to the most potent antibiotics.

Most research on antimicrobial resistances (AMRs) has focused on inland systems with comparatively little effort directed toward water habitats. Marine and particularly coastal environments can function as transmission foci for potentially pathogenic bacteria (PPB) because of the concentrated aggregations of bacteria from different sources, both marine and terrestrial, where environmental, human, and/or animal-related bacteria can coexist, at least temporarily (Prestinaci *et al.*, 2015). This mixing and the highly altered condition of coastal marine ecosystems i.e. anthropization, contaminants, pharmaceutical residues etc. can affect species interactions, select ARB, and trigger disease emergencies, both for animals and humans (Grigorakis *et al.*, 2011). The main risk to public health and aquaculture production is assumed to be the development of a reservoir of antibiotic-resistant genes that can be transferable to human or marine livestock pathogens, and thus cause the emergence of pathogens with newly acquired antibiotic-resistant determinants. In addition, aquaculture activities apply antibiotics directly to the water or mix them into foods, promoting the development of antibiotic-resistant bacteria in aquatic animals and the environment (Ashish *et al.*, 2022). Thus, the risk of infection with pathogenic antibiotic-resistant bacteria linked to the marine habitat or marine products is therefore a major world health challenge.

Vietnam is recognized as a country heavily affected by AMRs (Mckinn *et al.*, 2021). The main cause is the indiscriminate use of antimicrobials in animal production, accounting for about 70% of the total antimicrobial

consumption (Phu et al., 2022). In addition, Vietnam has become one of the biggest aquaculture producers in the world. Many aquaculture farms in Vietnam are highly connected, which can represent important reservoirs and key dissemination pathways of potential pathogens and ARGs. Nha Trang Bay is heavily impacted by anthropogenic inputs of urban and/or aquaculture origins, which are a good model for studying the AMRs circulated in sea coastal areas of Vietnam. A previous study highlighted the presence of antibiotics such as Tetracycline, Trimethoprim, Rifampicin, Nifuroxazide, and Colistin used in aquaculture operations in the Nha Trang Bay (Hedberg *et al.*, 2018). Given the potential for antimicrobial-resistant bacteria and genes to transfer among humans, animals, and the environment, a One Health strategy is crucial. This study investigates the distribution of potential pathogenic antibiotic-resistant bacteria in Nha Trang Bay, Vietnam.

## MATERIALS AND METHODS

### Bacterial isolation and identification

Marine water and sediment samples were collected at 12 sites across three transects including R – Recovery area (R1, R2, R3 and R4); U – Urban area (U1, U2, U3 and U4) and F - Aquaculture Farm area (F1, F2, F3 and F4) from geographical coordinates (between 12°18'97.7" N to 12°19'64.0" N latitude and 109°31'55.2" E to 109°21'82.5" E longitude) spanning the entire coastal area of Nha Trang Bay.

At each station, surface water was collected in 500 ml sterile polypropylene bottles, while sediment samples were stored in 50 ml Falcon tubes with screw caps and placed immediately in an ice box. Bacteria were then cultured on selective media (Merck, Germany) including MacConkey Agar, *Aeromonas* and Marine Agar. Pure isolates were identified at species level using the MALDI Biotyper® Sirius One IVD System (Bruker Daltonics, Germany).

### Antibiotic susceptibility testing

Antimicrobial susceptibility testing was conducted using the Kirby Bauer disc diffusion method on Mueller–Hinton Agar plates, according to the Clinical and Laboratory Standard Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Specifically, for the Enterobacteriaceae strains, six groups of antibiotics including Penicillin (Amoxicillin + clavulanic acid; Ticarcillin; Piperacillin; Amoxicillin; Ticarcillin + Clavulanic acid; Piperacillin + Tazobactam), Cephalosporins (Cefotaxime; Cefoxitin; Cefepime; Ceftazidime), Carbapenems (Ertapenem; Imipenem), Aminoglycosides (Tobramycin; Gentamicin), Fluoroquinolones (Ciprofloxacin; Levofloxacin), and Monobactams (Aztreonam) were selected. For the *Pseudomonas* strains, the groups of antibiotics tested included Cephalosporins (Cefepime), Carbapenems (Imipenem; Meropenem), Aminoglycosides (Gentamicin), Fluoroquinolones (Ciprofloxacin; Levofloxacin), and Monobactams (Aztreonam). For the *Aeromonas* and *Acinetobacter* strains, antibiotic groups were selected including Penicillin (Ticarcillin; Pipe + Tazobactam), Cephalosporin (Ceftazidime; Cefepime), Carbapenem (Imipenem; Meropenem), Aminoglycosides (Tobramycin; Gentamicin), and Fluoroquinolones (Cotrimoxazole; Ciprofloxacin; Levofloxacin).

### Multidrug-resistant bacteria and Multiple Antibiotic Resistance index

Multidrug-resistant bacteria were defined as bacteria resistant to more than two classes of antibiotics among all tested antibiotics. The multiple antibiotic resistant index was calculated using the formula below (Reverter *et al.*, 2020).

$$\text{MAR} = \frac{\text{(Number of antibiotics to which the isolate was resistance to (a))}}{\text{Total number of antibiotics tested (b)}}$$

If the MAR index  $\geq 0.2$ : high risk source of antibiotic contamination

### Environmental DNA extraction

Environmental DNA (eDNA) of soil and water samples was extracted using the DNeasy PowerSoil Pro kit (QIAGEN, Germany) according to the manufacturer's guideline. The eDNA samples were examined using 1% agarose gel electrophoresis and were visualized using Benchtop UV Transilluminators (Analytik Jena, Germany). The purity and quality of eDNA were determined using Nanodrop 2000 spectrophotometers (Thermo Fisher, USA).

### Detection of antibiotic resistant genes

For each eDNA sample, 19 AMRs including *sul1*, *sul2*, *tetA*, *tetQ*, *tetB*, *tetM*, *blaKPC*, *blaOXA-48*, *blaVIM*, *blaNDM*, *blaIMP*, *mcr-1*, *qnrA*, *vanA*, *mecA*, *ermB*, *quepA* and *rmfB* were screened by PCR as previously described. The PCR amplicons were examined on a 1% agarose gel stained with Red Safe (Intron, Korea) and were visualized under Benchtop UV Transilluminators (Analytik Jena, Germany).

RESULTS AND DISCUSSION

Distribution of potential pathogenic bacteria

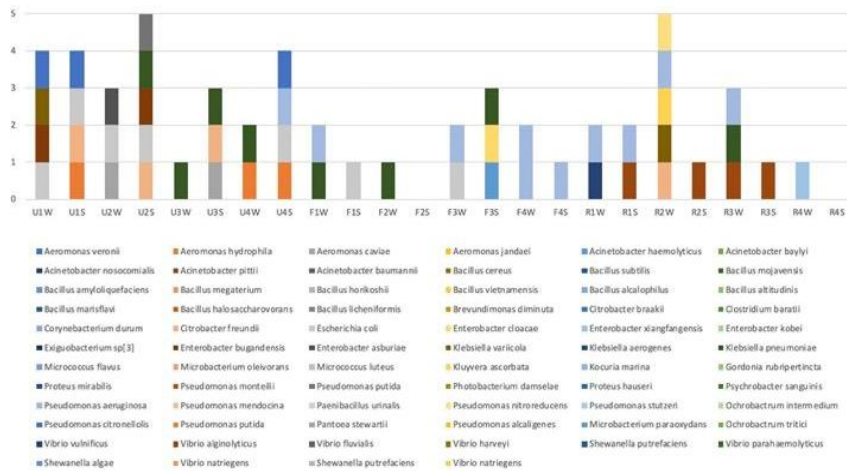


Figure 1. Distribution of potential pathogenic bacteria across different sampling areas

102 out of 156 bacterial isolates were successfully identified using MALDI-TOF MS method, in which 53 isolates were identified as potential pathogenic species (Figure 1). These pathogens were found in all surface water samples, while they were not detected in 2/12 sediment samples. Pathogens were found in high abundance in urban areas (n=26, 49.0%), followed by Recovery areas (n=15, 28.3%) and Farm areas (n=12, 22.7%). The proportion of pathogenic bacteria in water samples was slightly higher than in sediment samples (52.3% and 47.7%, respectively). Overall, the most common potential pathogenic bacteria include *Pseudomonas aeruginosa* (20.6%), *Klebsiella pneumoniae* (15.1%), *Escherichia coli* (13.2%) and *Citrobacter freundii* (7.5%). These pathogens were distributed in the Nha Trang Bay. Notably, no pathogenic species were found to be common between water and sediment samples. Our findings indicate that Nha Trang Bay is contaminated with diverse potential pathogenic bacteria linked to hospital-acquired and community-acquired infections, highlighting the health risks for both humans and animals.

Phenotypic antibiotic-resistant profile of potential pathogenic bacteria

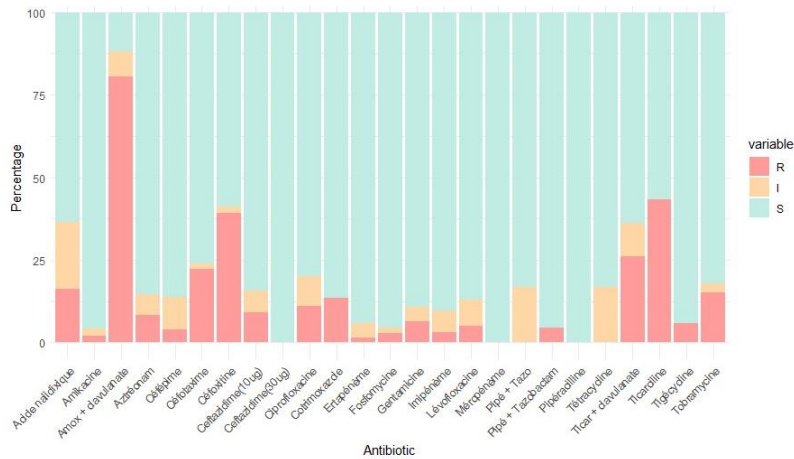


Figure 2. Proportion of antibiotic resistance

Most of the potential pathogenic bacteria were resistant to amoxicillin + clavulanic acid (80%), followed by cefoxitin, ticarcillin, nalidixic acid and cefotaxime (Figure 2). In fact, beta lactams and tetracycline are important antibiotic groups commonly used in medicine as well as in veterinary and in aquaculture as a feed additive and preventive treatment (Sodhi *et al*, 2020). Therefore, the resistance proportion to these antibiotics is very high in both clinical settings and aquacultures. Luckily, these pathogens were completely susceptible to ceftazidime, piperacillin and meropenem (100%), and were highly susceptible to amikacin, ertapenem and tigecycline (>90%). These antibiotics are very potent and the resistance rate is often very low, particularly in the environmental bacteria. This finding underlines the high risk of dissemination of these resistant pathogens in a large scale, particularly in the coastal areas around Nha Trang Bay.

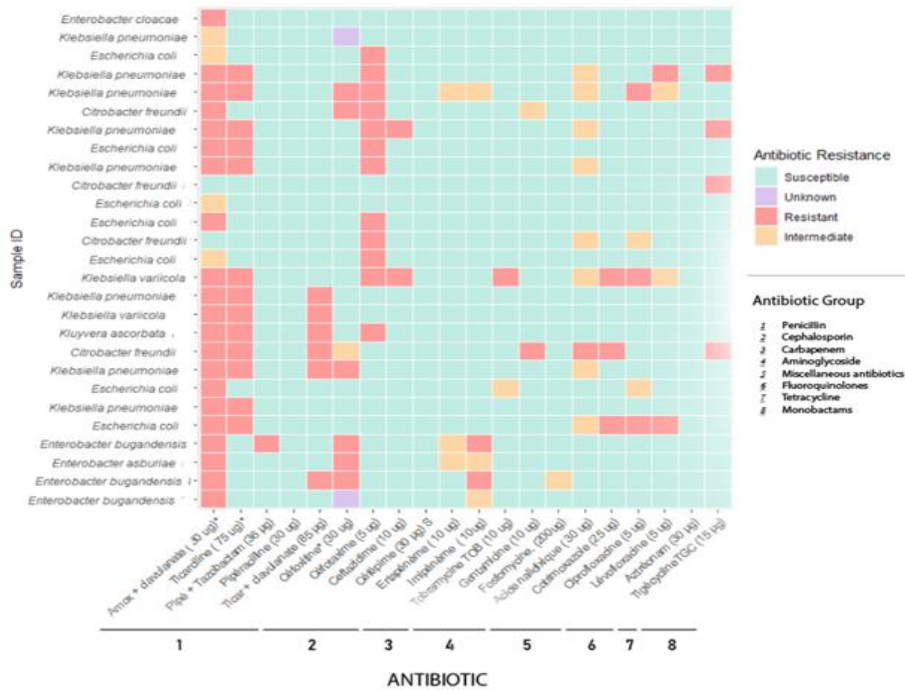


Figure 3. Antibiotic-resistant profile of *Enterobacteriaceae* species

25/27 (92.6%) *Enterobacteriaceae* bacteria were resistant to at least one antibiotic, while only two *Citrobacter freundii* isolates were completely susceptible to the antibiotics tested (Figure 3). Of the antibiotic-resistant phenotypes, 15 isolates displayed resistance to 3 – 7 antibiotics. The highly resistant bacteria were *Klebsiella variicola* and *Citrobacter freundii* which were resistant to seven antibiotics tested. Overall, the antibiotic-resistant profile of isolates was different within bacterial species suggesting that they acquired antibiotic resistance independently. For non- *Enterobacteriaceae* bacteria, the proportion of antibiotic resistance was lower than *Enterobacteriaceae*. These bacterial species including *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Acinetobacter haemolyticus*, *Acinetobacter pittii* were highly susceptible to antibiotics tested. Recent studies demonstrated that the occurrence of antibiotic-resistant bacteria is increasing in aquatic environments (Reverter *et al.* 2020, Singh *et al.* 2022). The levels of Nha Trang Bay exposure to antibiotics and their correlation to the evolution and spread of resistant bacteria need to be further investigated.

**Distribution of multiple-antibiotic resistant bacteria**

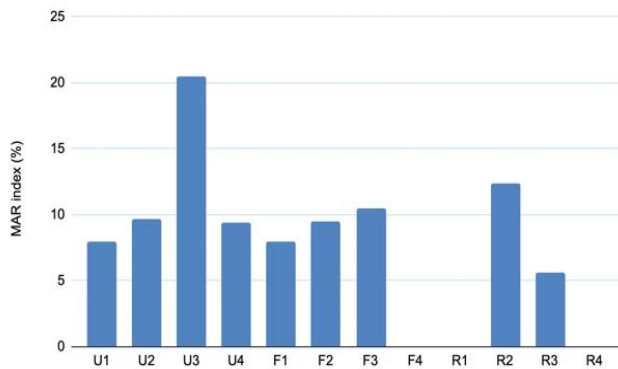
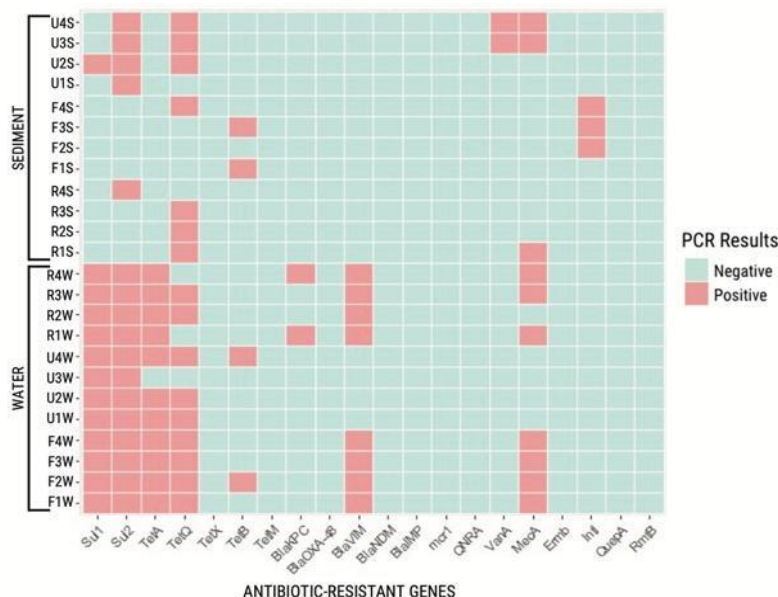


Figure 4. MAR index represented proportion of multiple-antibiotic resistant bacteria distributed in the Nha Trang Bay

High proportion of multiple-antibiotic resistant bacteria was found in urban and aquaculture farm areas, compared to the recovery area. The Urban transect exhibited the highest MAR bacteria rates, with all four sampling locations exceeding 8%, particularly, an urban station had the MAR index of over 20% (Figure 4), indicating a high density of multidrug resistance bacteria in this area. In the fish farm transects, the MAR index was also high, ranging from 8% to 10.5%, suggesting that the rapid emergence of multidrug-resistant bacteria in these areas under the high antibiotic pressure from aquaculture activities. Finally, multidrug-resistant bacteria were detected in two transects of recovery areas, underlying the transmission dynamic of resistant bacteria beyond the Nha Trang Bay.

## Distribution of antibiotic-resistant genes



**Figure 5. Occurrence of antibiotic-resistance genes in the marine environment of Nha Trang Bay**

In this study, 9 antibiotics-resistant genes including *su1*, *su2*, *tetA*, *tetQ*, *tetB*, *blaKPC*, *blaVIM*, *vanA* and *mecA* were detected in the marine environment of Nha Trang Bay (Figure 5). In concordance with studies in the world, *su1*, *su2*, *tetA* and *tetQ* were the most common ARGs in the environment (Singh *et al.* 2022, Xu *et al.* 2022). It is important to note that this region has a high density of aquaculture that is one of the most important sources of antibiotics released into the marine environment. This could possibly be linked to the widespread use of sulfonamides, tetracycline and beta lactams and resulting selective pressure for the corresponding ARGs. Notably, these antibiotics-resistant genes were mainly circulated in surface water (8 genes) compared to sediments (6 genes), in which *su1*, *su2*, *tetQ*, *tetB*, and *mecA* were shared between two habitats. In addition, the class 1 integron gene (*int1*) was only detected in sediments at fish farm stations. This finding suggests that *int1*-1 may facilitate the proliferation and propagation of some antibiotic-resistant genes in this region.

## CONCLUSION

In general, high prevalence of multidrug-resistant bacteria along with the abundance of antibiotic-resistant genes was detected in the water and sediment samples, suggesting that the Nha Trang Bay is an important reservoir for emergence and dissemination of AMRs. Our findings may be useful for developing effective strategies to control ARG contamination in the sea coastal area.

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## NGHIÊN CỨU VI KHUẨN KHÁNG KHÁNG SINH CÓ KHẢ NĂNG GÂY BỆNH PHÂN LẬP TỪ BIỂN NHA TRANG, VIỆT NAM

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### TÓM TẮT

Kháng thuốc (AMR) được công nhận là mối đe dọa nghiêm trọng đối với sức khỏe cộng đồng toàn cầu. Tuy nhiên, các nỗ lực nghiên cứu chủ yếu tập trung vào các hệ thống trên cạn, trong khi môi trường sông dưới biển ít được quan tâm hơn. Nghiên cứu này bước đầu xác định sự phổ biến và phân bố của vi khuẩn kháng thuốc ở biển Nha Trang, Việt Nam. Tổng cộng 53/106 (50%) loài vi khuẩn phân lập từ các mẫu nước và trầm tích được xác định là những mầm bệnh tiềm năng đối với người và động vật. Những mầm bệnh phổ biến nhất là *Pseudomonas aeruginosa* (20.6%), *Klebsiella pneumoniae* (15.1%), *Escherichia coli* (13.2%) và *Citrobacter freundii* (7.5%). Kháng sinh đồ thể hiện 60.4% (32/53) mầm bệnh đã kháng với tối thiểu một loại kháng sinh, trong đó 28.3% (15/53) chủng có kiểu hình đa kháng thuốc. Tỷ lệ kháng cao nhất đối với thuốc Amoxicillin/clavulanic acid (80%), tiếp theo đó là ticarcillin, cefoxitin, nalidixic acid, và cefotaxime. Cuối cùng, 9 gene kháng kháng sinh gồm *sul1*, *sul2*, *tetA*, *tetQ*, *tetB*, *blaKPC*, *blaVIM*, *vanA* và *mecA* đã được phát hiện. Những kết quả nghiên cứu này cho thấy Vịnh Nha Trang đóng vai trò quan trọng đối với việc bùng phát và phát tán các chủng vi khuẩn đa kháng thuốc trong môi trường biển, tiềm ẩn rủi ro đến an toàn thực phẩm, sức khỏe người và động vật.

**Từ khóa:** Vi khuẩn gây bệnh, đa kháng thuốc, gen kháng kháng sinh, môi trường biển, Vịnh Nha Trang.

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