CMU Journal of Science
The Official Scientific Journal of Central Mindanao University
ISSN Print: 0116-7847 js.cmu.edu.ph ISSN Online: 2704-3703

Research Article

Beta-Lactam- and Aminoglycoside-Resistant *Vibrio parahaemolyticus* in Marine Food Fish from Four Public Markets in Bukidnon Province, Philippines

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A B S T R A C T.

Vibrio parahaemolyticus is recognized for its pathogenic potential frequently associated with consumption of contaminated seafood, particularly fish. Although fish constitute a dietary staple in the Philippines, comprehensive data on the prevalence, antimicrobial resistance, and evolutionary relationships of *V. parahaemolyticus* remain scarce. This study assessed the presence of *V. parahaemolyticus* in three commonly consumed marine fishes (*Thunnus albacares*, *Rastrelliger brachysoma*, and *Dussumieria elopsoides*) sourced from public markets in Bukidnon Province using *toxR* gene amplification. Antimicrobial resistance (AMR) was determined for β-lactam and aminoglycosides, and antimicrobial resistant genes (ARGs) were evaluated targeting *blaCARB*, *blaCARB17*, *strA*, and *strB* genes, through the Kirby-Bauer method and PCR, respectively. Phylogeny was determined comparing *toxR* gene sequences using the NJ method at 1,000 bootstraps. Out of the nine isolates, seven have demonstrated resistance to ampicillin, three to ceftazidime, and seven to gentamicin. The *blaCARB* gene was identified in four isolates, confirming phenotypic resistance to ampicillin and ceftazidime. However, no isolates are identified carrying *strA* and *strB* genes despite phenotypic resistance to aminoglycoside. BLAST-based analysis of *toxR* sequences confirmed high percent identity (99.67-100%) with reference *V. parahaemolyticus* sequences in the NCBI database, validating species-level identification. Phylogeny indicated 83-100% sequence similarity among isolates, reflecting intra-species genetic diversity. Findings document the presence of β-lactam- and aminoglycoside-resistant *V. parahaemolyticus* in marine food fish, highlighting the necessity for enhanced seafood safety protocols. As one of the most common seafood-borne pathogens, the presence of this bacterium underscores the importance of ongoing AMR surveillance to mitigate food safety risks, particularly for consumers of raw fish.

Keywords: *Vibrio parahaemolyticus*, antimicrobial resistance, beta-lactam, aminoglycoside, public markets, marine fishes

Citation: Remon, J., Elumba, Z., Cempron, M. G., Dela Cruz, R., & Toledo, J. M. (2026). Beta-Lactam- and Aminoglycoside-Resistant Vibrio parahaemolyticusin Marine Food Fish from Four Public Markets in Bukidnon Province, Philippines. CMU Journal of Science, 30(1), 94

Academic Editor:
Dr. Frolan Aya

Received: Dec. 12, 2025
Revised: May 13, 2026
Accepted: June 5, 2026
Published: July 2, 2026

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INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium that naturally inhabits marine and estuarine waters (Fang et al., 2023), and a major seafood-borne pathogen that causes infections, particularly gastroenteritis linked to undercooked or raw seafood consumption (Bourdonnais et al., 2024; Tada et al., 1992). Gastroenteritis infection caused by this bacterium is generally self-limiting, and could be treated with antibiotics, but their misuse or abuse can lead to the emergent of resistant strains (Bourdonnais et al., 2024; Chiou et al., 2015; Diemert, 2006; Su & Liu, 2007). Marine food fish are a common vehicle for the transmission of *V. parahaemolyticus* to humans. Fish is a dietary staple and an affordable source of protein in the Philippines, with the fisheries sector contributing approximately 1.5% to the national GDP (SEAFDEC, 2022). Despite its nutritional and economic importance, an information gap remains regarding *V. parahaemolyticus* contamination in marine food fish sold in local markets, as well as antimicrobial resistance profile and genetic relationship between *V. parahaemolyticus* isolates from different market sources.

The *V. parahaemolyticus* pathogen possesses several virulence factors, including the primary virulence factors thermostable direct hemolysin (TDH), and TDH-related hemolysin (TRH), both of which exhibit hemolytic activity (Honda et al., 1992; Honda & Iida, 1993; Kishishita et al., 1992). Additionally, it harbors three distinct type III secretion systems (T3SS1, T3SS2α, and T3SS2β) that contribute to enterotoxic and/or cytotoxic effects (Ham & Orth, 2012). It has been demonstrated that these virulence factors are regulated by the ToxR protein composed of 20 genes that encode products promoting intestinal colonization, toxin production, and host survival (DiRita et al., 1991). Specifically, the *toxR* gene, a regulatory gene of the toxin operon in *Vibrio* and part of the ToxR protein, is involved in the regulation of virulence genes (Kim et al., 1999; Sechi et al., 2000). Detection of virulence genes in *V. parahaemolyticus* is primarily used for determining pathogenic strains. However, *toxR* gene, aside from its primary function as a regulator for the expression of virulence factors, can be used to identify both pathogenic or non-pathogenic *V. parahaemolyticus* (Sechi et al., 2000). Although *toxR* is not directly responsible for encoding major hemolysins, its regulatory influence over virulence-associated pathways renders it a reliable molecular indicator of pathogenic potential (Letchumanan et al., 2014). Comparative sequence analyses have revealed that while *toxR* is conserved across multiple *Vibrio* species, it exhibits species-specific polymorphisms that enable its use as a molecular marker for species-level identification. For instance, the gene sequence in *V. parahaemolyticus* demonstrates sufficient divergence from homologous sequences in closely related species such as *Vibrio alginolyticus* and *Vibrio cholerae*, thereby facilitating its application in PCR-based diagnostics for accurate detection and differentiation (Kim et al., 1999; Montieri et al., 2010).

In addition to the virulence factors of *V. parahaemolyticus*, the rise and emergence of antimicrobial resistance (AMR) in *V. parahaemolyticus* strains is becoming a major public health concern for countries with seafood-consuming populations. AMR is the ability of bacteria to survive and grow despite exposure to drugs intended to inhibit or eliminate them (FAO, 2024). The evolution of AMR in *Vibrio* species, including *V. parahaemolyticus*, is primarily linked to the widespread use of antibiotics in aquaculture and clinical settings (Gould, 2016). Evidence of AMR has been detected in some clinical and environmental isolates of *V. parahaemolyticus*, where excessive antibiotic use promotes the positive selection of antimicrobial resistance genes (ARGs), thereby accelerating their spread among microbial populations (Zhang et al., 2022). Like other bacteria, the mechanism of antibiotic resistance developed by *V. parahaemolyticus* can be classified as either intrinsic or acquired through horizontal gene transfer (HGT) (Dutta et al., 2021). However, genetic determinants that confer antimicrobial resistance in bacteria are also found in plasmids (Bennett, 2008; Szczepanowski et al., 2009), which serve as mediators for the transfer of antibiotic-resistant genes via HGT (Okamoto et al., 2009; Wang et al., 2024). Molecular-level assessment of AMR in *V. parahaemolyticus* is a vital component of AMR surveillance that could offer insights into the genetic determinants and mechanisms that drive resistance.

This study aimed to characterize and confirm identification of the putative *V. parahaemolyticus* isolates from market marine fish samples through *toxR* gene detection, evaluate the antimicrobial resistance and molecular phylogeny of antimicrobial-resistant *V. parahaemolyticus* isolates and analyze their antimicrobial resistance genes.

METHODOLOGY

Screening of Isolates

Presumptive *V. parahaemolyticus* isolates were previously obtained from yellowfin tuna (*Thunnus*

albacares), short-bodied mackerel (*Rastrelliger brachysoma*), and sardine (*Dussumieria elopsoides*) samples collected from four public markets in Bukidnon Province, Philippines, namely Valencia City Central Market, Maramag Public Market, Malaybalay City Public Wet Market, and Quezon Central Market. The fish samples were selected based on the top fish species consumed by the local provincial population. For each market, 3 samples per species were collected between the months of July to September of 2024, during rainy seasons. Of the 36 fish samples collected, at least 35 presumptive isolates showing round-green colonies were obtained and were subjected for further characterization. However, only a few isolates were selected for *toxR* and AMR genes sequencing. These isolates were selected based on characteristics, particularly on the presence of *toxR* genes, and virulence genes (*tdh* and *trh*) upon PCR amplification and antimicrobial resistance phenotypes, ensuring variation based on locations, and were re-cultured on thiosulfate-citrate-bile salts-sucrose (TCBS, HiMedia®) agar plates. A total of nine isolates were recovered and subsequently screened using Gram staining to confirm their morphological characteristics, as curved rod-shaped and Gram-negative. Upon re-culture, each isolate was labeled based on their market and sample sources (Table 1).

Table 1. Labeling system for the isolates.

Isolate number	Market source	Sample source	Occurrence per sample	Code
1	Valencia City	Sardine	1	CVH3.1
2	Valencia City	Short-bodied mackerel	1	CVM1.1
3	Maramag	Short-bodied mackerel	1	MMT1.1
4	Malaybalay City	Sardine	1	CMH3.1
5	Malaybalay City	Short-bodied mackerel	1	CMM1.1
6	Malaybalay City	Yellowfin tuna	1	CMT1.1
7	Quezon	Sardine	1	MQH2.1
8	Quezon	Short-bodied mackerel	1	MQM3.1
9	Quezon	Yellowfin tuna	1	MQT1.2

The isolates were labeled based on the initial of the common name of the fish, a sample collection number specific to the fish species, and an isolate identifier for each purified strain from the sample. For example, in the code CVM1.1, "CV" indicates the sampling site (City of Valencia), "M" corresponds to the fish common name (Mackerel), "1" designates the first mackerel sample out of three collected at that site, and ".1" denotes the first distinct isolate purified from this sample. For the case of sardine, "H" was used, since the isolates were obtained prior to the sample identification, pre-identified as "Herring."

Molecular Characterization

DNA isolation for each isolate was carried out using the boiling lysis method described by Jones et al. (2012). The DNA purity was measured using the standard A260/A280 ratio to align with the acceptable values between 1.8 to 2.0. PCR amplification of the *toxR* gene was performed using the *V. parahaemolyticus toxR*-specific primer, with a target yield of 368 bp PCR product (Parthasarathy et al., 2021). The primer sequences used were as follows: F 5′-GTC TTC TGA CGC AAT CGT TG-3′ and R 5′-ATA CGA GTG GTT GCT GTC ATG-3′ (Kim et al., 1999). Each 20 µL PCR reaction consisted of 0.1 µL of 0.5 U Ex Taq polymerase, 2.0 µL of 10X PCR reaction buffer (Ex Taq Buffer; Takara, Japan), 1.6 µL of 0.2 mM each dNTP, 5.0 µL of DNA Template, 0.24 µL each of 0.03 µM primer, and 10.82 µL of sterile ultra-pure water. PCR amplifications were performed in a T100TM thermal cycler (Bio-Rad; California, United States), with the following conditions: 20 cycles of amplification, consisting of denaturation (94 °C for 1 minute); annealing (63 °C for 1 minute and 30 seconds); and extension (72 °C for 1 minute and 30 seconds) (Kim et al., 1999). Sequencing was performed by Macrogen, Inc. (Seoul, South Korea), using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems; Massachusetts, United States) on an ABI PRISM 96-capillary 3730XL DNA analyzer. The BioEdit Sequence Alignment Tool was used to trim and align the DNA sequences obtained. The sequences were then analyzed for similarity using the nucleotide BLAST (BLAST-n) tool on the NCBI website, with the nucleotide collection (nr/nt) database selected as the target for sequence comparison. To elucidate for the phylogeny, *toxR* sequences were subjected to MEGA11 software by multiple sequence alignment using MUSCLE, and evolutionary relationships were determined using the neighbor-joining (NJ) method with 1,000 bootstrap replicates, with evolutionary distances

between sequences were calculated using the maximum composite likelihood method.

Antibiotic Susceptibility Profiling

The nine (9) isolates were subjected to antibiotic susceptibility testing using the Kirby-Bauer method against eight (8) antibiotics, and were incubated at 35 °C for 18 hours, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) document M45, 3rd edition, for *Vibrio* spp (CLSI, 2016). The 8 antibiotic discs were placed on MHA plates with a control disc containing 10 µL distilled water. The selected antibiotic discs (Bioanalyse®; Ankara, Türkiye) represented classes that inhibit bacterial cell wall synthesis, DNA synthesis, and protein synthesis, addressing different mechanisms of action (Elmahdi et al., 2016; Lopatek et al., 2018; Tabo et al., 2015). Four (4) antibiotics, to which some *V. parahaemolyticus* strains exhibit high resistance, were included: 10 µg ampicillin (AM10); 30 µg ceftazidime (CAZ30); 10 µg gentamicin (CN10); and 30 µg tetracycline (TE30). Another four (4) antibiotics were also included, where *V. parahaemolyticus* strains generally show high susceptibility: 10 µg meropenem (MEM10); 5 µg ciprofloxacin (CIP5); 1.25/23.75 µg trimethoprim-sulfamethoxazole (SXT25); and 30 µg chloramphenicol (C30). Multi-drug resistance among isolates was assessed following the definition of Magiorakos et al. (2012), where isolates that show non-susceptibility (i.e., resistant or intermediate) to ≥ 1 agent in ≥ 3 antimicrobial classes are considered multidrug-resistant (MDR).

The nine (9) isolates were screened for AMR genes based on phenotypic resistance patterns in antibiotic susceptibility tests, referencing previous studies on AMR genes within *V. parahaemolyticus* isolates. PCR amplification was conducted using synthesized oligonucleotide primers (Table 2) designed to target specific AMR genes associated with known resistance mechanisms. The PCR procedures and sequencing were performed by Macrogen, Inc. (Seoul, South Korea), with the same procedures for *toxR* sequencing mentioned earlier. For PCR amplification, DNA extraction was performed using InstaGene Matrix (Bio-Rad; California, United States). Since the annealing temperature for each primer was undetermined, gradient PCR amplification was followed by mixing DNA template with Taq polymerase MBAE-110 AxenTM H Taq PCR Master Mix (2X). Amplification was done using DNA Engine Tetrad 2 Peltier Thermal cycler (Bio-Rad; California, United States) to obtain ~500 bp for *blaCARB*, ~303 bp for *blaCARB17*, ~500 bp for *strA*, and ~500 bp for *strB*, with the following conditions: initial denaturation (95 °C for 5 minutes); followed by 35 cycles of denaturation (95 °C for 30 seconds); annealing at different temperatures (48 °C, 49.4 °C, 51.8 °C, 55.5 °C, 60 °C, 63.9 °C, 66.4 °C, and 68 °C for 30 seconds); and extension (72 °C for 1 minute); with a final extension (72 °C for 10 minutes). Gel electrophoresis was followed using 2 µL each of PCR DNA product in 1.5% agarose gel, stained with Gel Red, and run at 300 V for 20 minutes. Thermo Fisher Scientific (California, United States) GeneRuler DNA Ladder Mix (#SM0331) was used as a DNA marker.

Resistome Analysis

Table 2. Primer sets for the detection of antimicrobial resistance (AMR) genes.

AMR gene(s)	Primer sequence(s) (5′–3′)	amplicon size (~bp)	Reference(s)
blaCARB	F: 5′-GCT GAG AGC TCA TGA AAA AGT TA-3′ R: 5′-CGT AGG ATC CTT AAC TTT ATT TGT AGT GC-3′	~ 500	Wang et al. (2022), Zhang et al. (2024)
blaCARB17	F: 5′-ACC TTT GAT GGA AGA TA-3′ R: 5′-TCT AAC TTT CTT TGT AGT GCA-3′	~ 303	Hu et al. (2021), Li et al. (2016)
aph(3″)-Ib (*strA*)	F: 5′-TGG CAG GAG GAA CAG GAG G-3′ R: 5′-AGG TCG ATC AGA CCC GTG C-3′	~ 500	Zhang et al. (2024), Zhang et al. (2017)
aph(6)-Id (*strB*)	F: 5′-ATC GTC AAG GGG ATT GAA ACC-3′ R: 5′-GGA TCG TAG AAC ATA TTG GC-3′	~ 500	Zhang et al. (2024), Zhang et al. (2017)

RESULTS AND DISCUSSION

Phylogenetic Tree Analysis

The BLAST-based similarity analysis of the *toxR* sequences for each isolate revealed high sequence identity (99.67% to 100.00%), and E-values consistently

recorded at 0.00. In the context of BLAST (Basic Local Alignment Search Tool), the E-value represents the expected number of alignments with a given score that could occur by chance in a database of a particular size, wherein an E-value of 0.00 indicates an extremely low probability of random alignment, thereby signifying a highly significant match between the query and reference sequences. This statistical measure is significant in confirming the authenticity of sequence homology, especially when the percent identity is near-perfect (Altschul et al., 1990). Among the nine (9) isolates, eight exhibited 100.00% identity to established *V. parahaemolyticus* reference sequences in the NCBI nucleotide collection (nr/nt) database. The remaining isolate, MMT1.1, showed a slightly lower identity of 99.67%, yet still retained an E-value of 0.00, supporting the reliability and biological relevance of the match. Furthermore, query coverage values ranged from 99.00% to 100.00% across all sequences, indicating that nearly the entire length of each query sequence aligned with the reference, thus supporting accurate taxonomic identification of the isolates as *V. parahaemolyticus* (Table 3).

Table 3. Top BLAST-n hits for each isolate derived from *toxR*-based genomic DNA sequence analysis.

Scientific name	Accession number	Max score	Total score	Query cover	E-value	Percent identity
Isolate CMH3.1						
Vibrio parahaemolyticus	AB300873.1	551	551	100%	0.00	100%
Isolate CMM1.1						
Vibrio parahaemolyticus	CP046782.1	549	549	100%	0.00	100%
Isolate CMT1.1						
Vibrio parahaemolyticus	CP046782.1	551	551	100%	0.00	100%
Isolate CVH3.1						
Vibrio parahaemolyticus	CP078655.1	549	549	100%	0.00	100%
Isolate CVM1.1						
Vibrio parahaemolyticus	CP150902.1	520	520	100%	0.00	100%
Isolate MMT1.1						
Vibrio parahaemolyticus UCM-V49	CP007004.1	558	558	100%	0.00	99.67%
Isolate MQH2.1						
Vibrio parahaemolyticus	CP150902.1	551	551	100%	0.00	100%
Isolate MQM3.1						
Vibrio parahaemolyticus	CP078641.1	569	569	99%	0.00	100%
Isolate MQT1.2						
Vibrio parahaemolyticus	CP078611.1	562	562	100%	0.00	100%

The evolutionary relationships among various *Vibrio* species, based on the *toxR* gene sequences, with a focus on multiple isolates of *V. parahaemolyticus* (from this study), and *Photobacterium damselae* subsp. *damselae*, as the outgroup, are presented in Figure 1. The placement of the outgroup *Photobacterium damselae* confirms its status as the most distantly related species among those included in the phylogenetic tree, providing a reference point for inferring the evolutionary direction of the isolates and other *Vibrio* species.

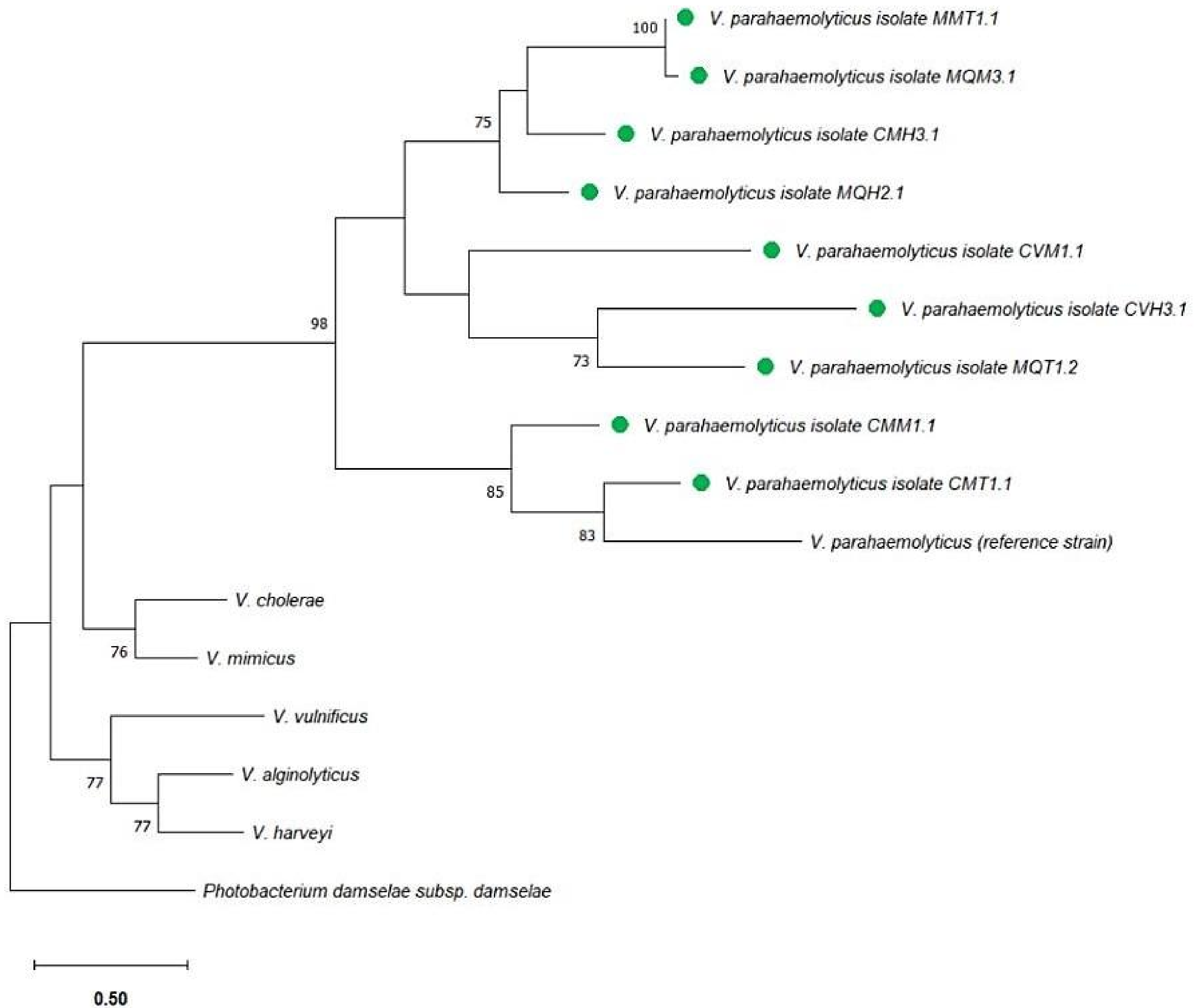

Figure 1. Phylogenetic tree illustrating the placement of *V. parahaemolyticus* isolates (indicated by green bullets), constructed using sequences of the *toxR* gene. The tree includes various *Vibrio* species for comparative analysis, with *Photobacterium damselae* subsp. *damselae* serving as the outgroup.

Among the five *Vibrio* species included in this analysis, *V. harveyi* and *V. alginolyticus* appear to be closely related (77% bootstrap value), supporting their shared node. Further up the tree, *V. vulnificus* branches off next, suggesting a closer evolutionary relationship with the previously mentioned two species compared to the other species included in the analysis. On the other hand, *V. mimicus* and *V. cholerae* cluster together, having close evolutionary history reflected (76% bootstrap value) within their shared node, consistent with previous studies reporting genetic similarities between these two species, particularly in terms of ecological adaptations (Halder et al., 2022).

The largest clade in the tree is composed of nine *V. parahaemolyticus* isolates (from this study) and a GenBank-derived reference strain of *V. parahaemolyticus* (GenBank Accession Number: AB029915.1) from NCBI. Strains in this clade have high genetic similarity, which is evident in their bootstrap values of 73-100%. Wherein, the reference strain closely aligns with the isolate CMT1.1 at 83% bootstrap value. Within the same cluster, isolate CMM1.1 diverges slightly from the previously mentioned pair at 85%. Further branching shows that isolates MQT1.2, CVH3.1, and CVM1.1 form a subcluster (≥73%). Isolates MQH2.1 and CMH3.1 are closely positioned higher than the previously mentioned isolates, and isolates MMT1.1 and MQM3.1 have the highest similarity (100%), indicating a well-supported genetic similarity within their *toxR* sequences. The phylogenetic tree topology likely reflects consistent bootstrap support

across branches, affirming the reliability and stability of the inferred phylogenetic relationships among isolates. This interpretation is further supported by a finding that bootstrap proportions of ≥70% generally correspond to a probability of ≥95% that the associated clade represents a true evolutionary relationship (Hillis & Bull, 1993). Thus, the presence of high bootstrap values across the branches of the phylogenetic tree further strengthens the reliability and validity of the inferred clades. Moreover, the close clustering observed between the GenBank-derived *V. parahaemolyticus toxR* sequences and the isolates obtained in this study suggests a high degree of genetic conservation within this group despite having differences in their environmental sources. This clustering may indicate that the isolates from the public markets share common contamination pathways, potentially from seafood samples or aquatic environments in Mindanao. Furthermore, the clustering pattern may reflect cross-contamination associated with market handling practices, such as processing, transportation, storage, and retail distribution, which could contribute to the spread of closely related *V. parahaemolyticus* strains across different marine fish samples. Similar observations were reported by Elhadi et al. (2004), who documented the occurrence of diverse potentially pathogenic *Vibrio* species in Malaysian seafood products, attributing contamination to poor hygiene and inadequate seafood marketing systems, where *V. parahaemolyticus* was detected across several shellfishes, suggesting previous claims. Likewise, Parthasarathy et al. (2021) reported that *V. parahaemolyticus* contamination in fishes was strongly influenced by environmental exposure and cross-contamination during market handling, particularly through contact with contaminated shellfish products. In spite of genetic variations, the proximity of the GenBank-derived *V. parahaemolyticus toxR* sequence to the isolates from Bukidnon markets, provides internal confirmation of accurate species identification. This highlights the consistency between the molecular characteristics of the isolates and the known genetic profile of *V. parahaemolyticus*, which is obtained in NCBI. On the other hand, the formation of separate clade for other *Vibrio* indicates that they may have followed different evolutionary paths. This inclusion provides valuable comparative insights into both the evolutionary relationships and genetic distinctions among members of the genus at the *toxR* sequence level. Since the *toxR* is commonly associated with species identification and regulatory functions in *Vibrio*, variation within this gene may serve as evidence of species-level divergence while also demonstrating conserved evolutionary relationships among closely related taxa (Sawabe et al., 2007). Previous studies have likewise demonstrated that phylogenetic clustering based on the *toxR* gene sequences can reveal ecological adaptation and evolutionary relatedness, particularly those associated with seafood and aquatic environments (Kim et al., 1999; Sawabe et al., 2007).

Furthermore, understanding the linkages between these clustered isolates and specific market-handling or supply-chain practices is important in bridging the gap between phylogenetic findings and practical public health implications. Such insights can guide regulators in implementing targeted interventions to mitigate contamination risks, thereby translating phylogenetic findings into actionable strategies to strengthen seafood safety. The *Vibrio* species derived in NCBI included in this study are recognized for their roles in seafood-associated infections and diseases affecting aquatic animals, seafood products, and consumers. Incorporating these *Vibrio* species into this analysis may provide a comparison for assessing how the *toxR* gene varies among species with distinct pathogenic profiles and ecological adaptations, as the regulatory gene of these pathogens (Miller & Mekalanos, 1988). By examining *toxR* gene sequence variations among different *Vibrio* species, a comparison can be made regarding how evolution has shaped differences in gene structure and function. This often reflects adaptation to specific environmental conditions and may also influence the virulence potential of each species (Kim et al., 1999). In contrast to this comparison, the 16S rRNA sequences of *V. parahaemolyticus* and *V. alginolyticus* share high similarity at 99%. However, their *toxR* gene sequences are more distinct, allowing *toxR* to be a better marker for species-level differentiation in *Vibrio* species (Montieri et al., 2010). Furthermore, the *toxR* gene functionally encodes a membrane-bound transcriptional regulator that modulates key virulence genes. This gene activates cholera toxin and toxin-coregulated pilus genes in *V. cholerae*, while it regulates hemolysin production in *V. parahaemolyticus*, which are thermostable direct hemolysin or TDH-related hemolysin (TDH/TRH), and type III secretion systems (T3SS1, T3SS2α, and T3SS2β) (Lin et al., 1993).

Sequence-based comparisons of the *toxR* gene may provide insights into the functional divergence of virulence regulation, helping to explain why certain *Vibrio* species are more frequently associated with human disease (Zhang et al., 2018). The phylogenetic relationship of these strains carries significant implications for both molecular epidemiology and public health surveillance in *V. parahaemolyticus*, as a leading cause of seafood-associated gastroenteritis. Moreover, mapping its genetic diversity is critical for identifying virulent strains and tracking contamination sources in aquaculture, processing chains (Ceccarelli et al., 2013), market settings as well.

Antimicrobial Resistance of the Isolates

Antimicrobial resistance profiling of the nine (9) isolates revealed a high level of resistance to β-lactam

antibiotics, including cephalosporins. Seven (7) isolates exhibited resistance to ampicillin (77.78%), while three (3) were resistant to ceftazidime (33.33%), consistent with previous studies reporting elevated β-lactam resistance in *V. parahaemolyticus* isolated from seafood (Elmahdi et al., 2016; Tan et al., 2020) and in aquaculture environments (Siddique et al., 2021). This observed resistance patterns may reflect frequent exposure of this pathogen to β-lactam antibiotics, where amoxicillin, cephalosporins, penicillin, ampicillin, cephalexin, cefradine, and cefotaxime (Lulijwa et al., 2020) have been widely used in aquaculture industry in several countries, such as Brazil, Italy, Turkey, Korea, and South Africa (Bondad-Reantaso et al., 2023; Fri et al., 2018; Helena Rebouças et al., 2011; Kang et al., 2017; Laganà et al., 2011; Ozaktas et al., 2012). In addition, seven (7) isolates exhibited resistance to the aminoglycoside antibiotic gentamicin (77.78%) used in this study (Table 4). Multidrug resistance was not observed among the nine isolates, as none exhibited resistance or intermediate resistance to more than one antibiotic in at least three different antimicrobial classes (Magiorakos et al., 2012).

Table 4. Phenotypic resistance observed in nine (9) isolates based on antibiotic susceptibility profiling interpreted using the CLSI M45 guidelines for Vibrio.

			β-lactams								
			Penicillin	**Cephem**	**Carbapenem**	**Aminoglycoside**	**Tetracycline**	**Fluoroquinolone**	**Folate pathway inhibitor**	**Phenicol**
Isolate name	**Fish source**	**Market source**	**AM**	**CAZ**	**MEM**	**CN**	**TE**	**CIP**	**SXT**	**C**
CVH3.1	Sardine	Valencia City	R	R	S	R	S	S	S	S
CVM1.1	Short-bodied Mackerel	Valencia City	S	I	S	R	S	S	S	S
MMT1.1	Short-bodied Mackerel	Maramag	I	S	S	I	S	S	S	S
CMH3.1	Sardine	Malaybalay City	R	I	S	R	S	S	S	S
CMM1.1	Short-bodied Mackerel	Malaybalay City	R	R	S	R	S	S	S	S
CMT1.1	Yellowfin Tuna	Malaybalay City	R	R	S	R	S	S	S	S
MQH2.1	Sardine	Quezon	R	I	S	R	S	S	S	S
MQM3.1	Short-bodied Mackerel	Quezon	R	S	S	R	S	S	S	S
MQT1.2	Yellowfin Tuna	Quezon	R	S	S	I	S	S	S	S

Abbreviations: AM, ampicillin; CAZ, ceftazidime; MEM, meropenem; CN, gentamicin; TE, tetracycline; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; C, chloramphenicol; R, resistant; I, intermediate; and S, susceptible.

Genomic Resistome

The genomic resistome was based on sequence analysis of isolates with phenotypic resistance to antibiotics employed in this study. The *blaCARB* gene was detected in four (4) out of seven (7) isolates (57.14%) with phenotypic resistance to ampicillin (CVH3.1, CMH3.1, CMT1.1, and MQT1.2), and two (2) out of three (3) isolates (66.67%) with phenotypic resistance to ceftazidime (CVH3.1 and CMT1.1). Despite the absence of detection in some isolates, this finding provides strong molecular evidence of β-lactam antibiotic resistance, demonstrated by phenotypic resistance, particularly to ampicillin and ceftazidime. The *blaCARB*-positive isolates, sourced from sardines and yellowfin tuna, may indicate that seafood can serve as a reservoir for antimicrobial-resistant bacteria, posing a potential public health risk. However, it is important to acknowledge some limitations in this study. The PCR primer sets used may not cover all variants of antimicrobial resistance genes, potentially missing some forms of resistance. In addition, the sample size of isolates might limit the generalizability of the results.

The *blaCARB* gene encodes a class A carbenicillin-hydrolyzing β-lactamase in bacteria that confers resistance by hydrolyzing β-lactam antibiotics, rendering them ineffective (Chiou et al., 2015). Recent genomic surveillance studies have shown that the gene is frequently detected in *V. parahaemolyticus* strains isolated from seafood-borne outbreaks. For instance, isolates from outbreaks in Huzhou, China, exhibited high resistance rates to ampicillin and often carried the *blaCARB* gene, especially among strains of the emerging serotype O10:K4. This may support the claims that the gene is intrinsic (Yan et al., 2024). However, this resistance may also be under selective pressure from the use of antibiotics in aquaculture and food production systems (Yan et al., 2024). Although not directly investigated, the co-occurrence of resistance to third-generation cephalosporins, such as ceftazidime, may suggest the presence of additional resistant determinants, likely acquired through the mobilization of

mobile genetic elements (MGEs) carrying multiple AMR genes (Singh et al., 2018).

On the other hand, the non-detection of *aph(3")-Ib* (*strA*) and *aph(6)-Id* (*strB*) genes in seven (7) isolates having phenotypic resistance to aminoglycosides may suggest that there could be alternative resistance mechanisms. These two genes typically encode aminoglycoside-modifying enzymes (AMEs), specifically phosphotransferases that inactivate antibiotics such as streptomycin through the process of phosphorylation. Their detection is common in Enterobacteriaceae and *Pseudomonas* spp., where they are often carried on MGEs (Jana & Deb, 2006). However, in *V. parahaemolyticus*, aminoglycoside resistance may not be mediated solely by *strA* and *strB*, which are the canonical AME genes, used in this study. *V. parahaemolyticus* biofilms can develop resistance to aminoglycosides through multiple non-genetic mechanisms. These include biofilm enclosure that physically limits antibiotic penetration, neutralization via anionic extracellular polymeric substances (EPSs), and regulatory responses that upregulate resistance-associated genes under antibiotic stress. This further suggest that resistance may be phenotypically expressed without the presence, or the PCR-detectable presence of canonical AME genes (Tian et al., 2023). Moreover, whole-genome sequencing of environmental *V. parahaemolyticus* strains has revealed a diverse resistome, including efflux pumps, permeability barriers, and other AME genes beyond *strA* and *strB*, such as *aac(3)-IIa* and *aadA* (Song et al., 2024), which have not been targeted by PCR amplification. The failure to detect *strA* and *strB* genes may also be due to sequence variation in primer binding regions, gene silencing, or low copy number, all of which can hinder amplification from genomic DNA (Sundin, 2000).

CONCLUSION

Results from this study affirm that the putative isolates obtained from public markets across Bukidnon Province in the Philippines, were *V. parahaemolyticus*, based on *toxR* gene sequence analysis using BLAST-n and molecular phylogenetic comparison with GenBank-retrieved *V. parahaemolyticus toxR* sequences. Despite geographical differences among market sites, phylogenetic analysis revealed that all isolates were genetically related, exhibiting sequence similarities based on bootstrap values, ranging from 73 to 100%. Furthermore, majority of the isolates demonstrated resistance to selected antibiotics, particularly those belonging to β-lactam and aminoglycoside classes. β-lactam resistance was especially evident in four (4) isolates in which the *blaCARB* gene was detected. However, ARGs are not detected in some β-lactam-resistant and aminoglycoside-resistant isolates, despite phenotypic observations. These discrepancies may be attributed to some limitations in the study, particularly that the PCR primer sets used may not cover all variants of antimicrobial resistance genes, potentially missing some forms of resistance. These findings could highlight a pressing threat to food safety and public health, further emphasizing the adaptive potential of potentially pathogenic *V. parahaemolyticus*. Nevertheless, the study has several limitations, which have not been given a focus, where isolates were pre-selected based on their phenotypic resistance, virulence associated characteristics, and the presence of *toxR* gene, thereby limiting the representation of environmental variability and the determination of the occurrence across individual markets and throughout Bukidnon Province. The findings in this study highlight a significant public health concern, particularly if not addressed effectively through an effective One Health approach. This study further suggests the urgent need for stricter veterinary oversight on antibiotic use in aquaculture and the enforcement of stronger sanitation and food safety regulations. Future studies should adopt a more comprehensive approach that integrates ecological, epidemiological, and molecular approach, by investigating contamination pathways, expanding sampling technique to include both wet and dry seasons, and conducting comparative genomic analyses, to ARGs and other virulence-associated determinants.

Author Contributions: JMR (conceptualization, formal analysis, investigation, methodology, visualization, writing - original draft, writing - review and editing); ZSE (conceptualization, data curation, formal analysis, methodology, project administration, supervision, validation, visualization, writing - original draft, writing - review and editing); MGIC (conceptualization, methodology, and visualization); JMST (conceptualization, methodology, validation, writing - review and editing); and RYDC (conceptualization, methodology, resources, validation, writing - review and editing).

Funding: This research was funded by the Department of Science and Technology – Science Education Institute (DOST – SEI) through the Project STRAND scholarship granted for the primary author.

Data Availability Statement: The data generated and analyzed during this study are included in this article. Any raw data supporting the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: The authors acknowledge the Department of Science and Technology – Science Education Institute (DOST–SEI) project STRAND for the scholarship granted for the primary author, the Institute of Biological Sciences at Central Mindanao University, Tuklas Lunas Development Center, Local Government Units of Malaybalay City, Valencia City, Maramag, and Quezon, in Bukidnon, Philippines. The authors also acknowledge the invaluable assistance, expertise, and guidance of Nekka A. Mondaga, Kristina Casandra Pava, Walter Clint Bayani, Dr. Michelle Suelo, and Dr. Glenda Doblas.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

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