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Research Article

# Molecular Typing of Vibrio Species Isolated from Sea Bass (*Dicentrarchus labrax*) and Detection of Antibiotic Resistance

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**ABSTRACT**

This study was conducted to isolate *Vibrio* species from sea bass, identify them using molecular methods, and determine their antibiotic resistance. In the research, 100 sea bass samples taken from fish farms in the Aegean region between May and September 2021 were examined. After the phenotypic and genotypic identification of the isolates obtained from the samples, antibiotic resistances were determined by the disk diffusion method, and antibiotic resistance genes were determined by multiplex PCR. In this study, 46 (46%) *Vibrio* spp. isolates were obtained from 100 sea bass samples by conventional and biochemical methods. The obtained 46 isolates were confirmed to be *Vibrio* spp. by 16S rRNA PCR. From 46 isolates, 22 (47.8%) isolates were identified as *V. alginolyticus*, 13 (28.2%) isolates as *V. harveyi*, 3 (6.5%) isolates as *V. parahaemolyticus*, 1 (2%) isolate as *V. vulnificus* and 7 (15%) isolates as *Vibrio* spp. by multiplex PCR. The highest resistance was found to ampicillin (84.8%) in the isolates in the antibiogram. All isolates were found to be susceptible to enrofloxacin and sulfamethoxazole-trimethoprim (100%). In isolates, the highest resistance gene was found to be trimethoprim resistance gene (63%), and the lowest resistance gene was found to be fluoroquinolone resistance gene (6.5%). In this study, it was determined that *Vibrio* species have an important role as a primary agent in fish diseases, molecular methods give more reliable results in identification, and there is single and multiple antibiotic resistance among isolates.

***Keywords:*** *Antibiotic resistance, identification, PCR, Vibrio spp.*

# Levreklerden (*Dicentrarchus labrax*) İzole Edilen Vibrio Türlerinin Moleküler Tiplendirilmesi ve Antibiyotik Dirençliliklerinin Belirlenmesi

**ÖZET**

Bu çalışma, *Vibrio* türlerinin levreklerden izolasyonu, moleküler yöntemlerle tanımlanması ve antibiyotik dirençlerinin belirlenmesi amacıyla yapılmıştır. Araştırmada Mayıs-Eylül 2021 tarihleri arasında Ege bölgesindeki balık çiftliklerinden alınan 100 levrek örneği incelendi. Örneklerden elde edilen izolatların fenotipik ve genotipik olarak tanımlanmasının ardından disk difüzyon yöntemiyle antibiyotik dirençleri ve multipleks PCR ile antibiyotik direnç genleri belirlendi. Araştırmada 100 levrek örneğinden konvansiyonel ve biyokimyasal yöntemle 46 (%46) *Vibrio* spp. izolatı elde edilmiştir. Elde edilen 46 izolatın16S rRNA PCR ile *Vibrio* spp. olduğu doğrulanmıştır. Multipleks PCR ile 22 (%47,8) izolatın *V. alginolyticus*, 13 (%28,2) izolatın *V. harveyi*, 3 (%6,5) izolatın *V. parahaemolyticus*, 1 (%2) izolatın *V. vulnificus*, 7 (%15) izolatın *Vibrio* spp. olduğu belirlenmiştir. Antibiyogramda en yüksek direncin ampisiline (%84,8) karşı geliştiği belirlenirken tüm izolatların enrofloksasin ve sulfametoksazol-trimetoprime (%100) duyarlı olduğu tespit edilmiştir. İzolatlarda en yüksek direnç geninin trimetoprim direnç geni (%63), en düşük direnç geninin ise florokinolon direnç geni (%6,5) bulunmuştur. Bu çalışmada *Vibrio* türlerinin balık hastalıklarda primer erken olarak önemli bir rolü olduğu ve identifikasyonda moleküler yöntemlerin daha güvenilir sonuç verdiği, izolatlar arasında tekli ve çoklu antibiyotik direnci bulunduğu tespit edilmiştir.

***Anahtar kelimeler:*** *Antibiyotik dirençliliği, identifikasyon, PCR, Vibrio spp.*

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## Introduction

Aquaculture has emerged as a critical sector in meeting the escalating global demand for affordable, high-quality protein, driven by rapid population growth. Technological advancements have further elevated the significance and expansion of aquaculture (Done et al., 2015). According to the United Nations Food and Agriculture Organization (FAO), global aquaculture production reached 114.5 million tons, valued at 263.6 billion US dollars in 2018. Of this production, 54.3 million tons comprised fish, with 47.0 million tons from freshwater and 7.3 million tons from marine fish farming (FAO, 2020).

In Türkiye, aquaculture began in the 1960s with *Cyprinus carpio* and rainbow trout (*Oncorhynchus mykiss*), gaining momentum with the introduction of sea bream and sea bass farming in the 1980s (Arslan & Yıldız, 2021). Currently, sea bream represents 35% and sea bass 26% of marine aquaculture in Türkiye, while rainbow trout is the dominant species in freshwater aquaculture (TÜİK, 2021).

The major bacterial diseases affecting cultured fish in Türkiye include Vibriosis (Candan, 1991; Onuk et al., 2018; Duman et al., 2023), Photobacteriosis (Çağırgan, 1993), motile Aeromonas septicaemia (Baran, 1980; Duman et al., 2018), Pseudomonas infection (Matyar et al., 2010; Duman et al., 2021), Mycobacteriosis (Korun et al., 2005; Urku et al., 2018), Streptococcosis (Akaylı et al., 2008), Rickettsia infection (Timur et al., 2005), and Flavobacteriosis (Yardımcı, 2011; Saticioglu et al., 2018; Saticioglu et al., 2019).

Vibriosis, caused by *Vibrio* species, is particularly problematic in aquaculture, affecting various aquatic organisms including fish, molluscs, crustaceans, rotifers, and corals (Chong et al., 2011; Gomez-Gil et al., 2014). *Vibrio* species are Gram-negative, motile, rod-shaped bacteria, forming smooth, cream-colored colonies, and are oxidase-positive, fermentative, and susceptible to many Vibriostatic agent O/129 (Toranzo and Barja, 1990; Alsina and Blanch, 1994; Yaman et al., 2003; Noga, 2010; Actis et al., 2011).

The challenge of vibriosis disease to sustainable aquaculture is significant, and is further compounded by interactions between host, pathogen and environmental stressors (Toranzo et al., 2005; Noga, 2010). The ability of *Vibrio* species to thrive independently in aquatic environments underscores the urgency of addressing bacterial diseases in aquaculture (Pridgeon and Klesius, 2012).

In Türkiye, *V. harveyi* has been isolated from sea bream and sea bass, *V. anguillarum* from rainbow trout, sea bass, cultured sea bream, and red coral fish, and *V. ordalii* from sea bream and sea bass. Additionally, *V. alginolyticus*, *V. scophthalmi*, and *V. logei* have been isolated from cultured sea bass, alongside other pathogenic bacteria (Timur et al., 2005; Demircan and Candan, 2006; Korun, 2006; Tanrıkul, 2007; Akaylı et al., 2008; Tanrıkul and Gültepe, 2011).

Globally, antibiotics (such as oxytetracycline, enrofloxacin, florfenicol, and sulfonamides) are extensively used in Türkiye to treat fish bacterial diseases. However, the misuse and overuse of antibiotics contribute to the emergence of antibiotic-resistant bacteria and diminishing antibiotic efficacy over time (Colquhoun et al., 2007; Rodgers, 2009). Hence, accurate and prompt diagnosis of fish diseases and appropriate treatment selection are crucial (Kırkan et al., 2006; Akşit and Kum, 2008; Boran et al., 2013).

The aim of this study is to identify *Vibrio* species isolated from sea bream using traditional and molecular methods and to determine their antibiotic resistance and antibiotic resistance genes.

## Materials and Methods

### *Samples*

The research material consists of specimens of sea bass (*Dicentrarchus labrax*) raised in cage systems at aquaculture facilities along the Aegean Region coast. Sampling was conducted between May and September 2021, when water temperatures increase and fish losses due to diseases increase. A total of 100 samples were collected from suspected sea bass with lesions found dead in the cage systems of these farms and transported under a cold chain to the diagnosis laboratory of the Department of Microbiology.

### *Phenotypic Isolation and Identification of Vibrio Isolates*

Samples were taken from the internal organs (liver, spleen, kidney) of naturally infected and dead sea bass. Initially, blood agar with 1.5% NaCl (Merck, Germany) and MacConkey agar (Merck, Germany) were inoculated with the samples and incubated at 25°C for 24 hours. Then, round, smooth, semi-transparent, or cream-colored colonies with haemolysis were detected on blood agar, and transparent colonies on MacConkey agar in equivalent petri dishes were selected for Gram staining. The selected colonies were stained with Gram staining (Merck, Germany), and Gram-negative comma-shaped bacteria were used for further analysis. Catalase tests were initially performed on colonies determined to be Gram-negative. Oxidase tests (Merck, Germany) were performed on colonies that tested positive for catalase. Colonies showing a positive reaction in the oxidase test were subjected to the Vibriostatic agent O129 (Bio-Rad, USA) resistance test and incubated at 30 °C for 24 hours. Strains showing inhibition zones around the Vibriostatic agent O129 disks were considered sensitive. Colonies identified as sensitive were passaged onto TCBS agar (Merck, Germany) and incubated at 25 °C for 24 hours. Colonies observed in yellow and green colours were recorded as *Vibrio* spp. and stored at -20 °C in Brain Heart Broth (Merck, Germany) supplemented with 20% glycerol (Merck, Germany) and 1.5% NaCl (Thompson et al., 2004).

**Table 1.** Primer sequences and targeted amplicon sizes used in PCR analysis

| Target *Vibrio* Species | wPrimer Name | Primer Sequence (5'-3')[a] | Primer conc. (µM) | Amplicon size (bp) |
|---|---|---|---|---|
| *Vibrio* spp. | VG C2694352F46<br>VGC2694352R734 | GTCARATTGAAAARCARTTYGGTAAA-GG<br>ACYTTRATRCGNGTTTCRTTRCC | 1 | 689 |
| *V. parahaemolyticus* | VP 1155272F<br>VP 1155272R | AGCTTATTGGCGGTTTCTGTCGG<br>CKCAAGACCAAGAAAAGCCGTC | 0.24 | 297 |
| *V. cholerae* | VC C634002F<br>VC C634002R | CAAGCTCCGCATGTCCAGAAGC<br>GGGGCGTGACGCGAATGATT | 0.24 | 154 |
| *V. vulnificus* | VV2055918F79<br>VV 2055918R | CAGCCGGACGTCGTCCATTTTG<br>ATGAGTAAGCGTCCGACGCGT | 0.4 | 484 |
| *V. alginolyticus* | VA 1198230F<br>VA 1198230R | ACGGCATTGGAAATTGCGACTG<br>TACCCGTCTCACGAGCCCAAG | 0.1 | 199 |
| *V. mimicus* | VMC727581F<br>VMC727581R | ATAAAGCGGGCTTGCGTGCA<br>GATTTGGRAAAATCCKTCGTGC | 0.8 | 249 |
| *V. harveyi* | VH-4F<br>VH-7R | GTGATGAAGAAGCTTATCGCGATT<br>CGCCTTCTTCAGTTAACGCAGGA | 0,5 | 601 |

[a] Mixed base: K = G + T; R = A + G; Y = C + T; N = A + C + G + T

*Phenotypic Identification of Vibrio Isolates with BD Phoenix™*

In this study, *Vibrio* spp. suspected isolates were identified using the BD Phoenix (Becton Dickinson, USA) device. Fresh cultures of 24 h pure *Vibrio* spp. isolates on tryptic soy agar (Merck, Germany) were prepared in suspension with ID broth available in glass tubes, adjusted to McFarland 0.5 colony density. The BD Phoenix NMIC/ID-433 panel kit was used for Gram-negative bacterial isolates. The diagnosis was made using separate panels for each sample. ID Broth suspension tubes prepared separately for each sample were placed in the device for bacterial identification. Biochemical identification data obtained from the device were evaluated.

*Genotypic Identification*

*DNA isolation of Vibrio isolates*

*Vibrio* spp. colonies were passaged onto TSA agar (Merck, Germany) supplemented with 1.5% NaCl and incubated at 25 °C for 24 h. DNA was extracted from the fresh cultures with a DNA extraction kit (MagAttract® HMW DNA Kit, Qiagen, Germany).

*Primer sequences used for identification of Vibrio species*

The primer sequences used for the PCR analysis to detect *Vibrio* genus and *Vibrio* species in the study were designed by the manufacturer as specified in the studies by Kim et al. (2014, 2015) (Table 1). In the PCR analysis, the following standard strains were used as positive controls: *Vibrio alginolyticus* ATCC 17749, *Vibrio cholerae* ATCC 39050, *Vibrio harveyi* ATCC 33842, *Vibrio mimicus* ATCC 33653, *Vibrio parahaemolyticus* ATCC 17802, *Vibrio vulnificus* ATCC 27562.

*16S rRNA PCR analysis*

The DNA obtained from *Vibrio* spp. isolates were subjected to 16S rRNA analysis. For this purpose, a PCR mixture was prepared with a total volume of 25 µl containing 25 ng of sample DNA, 200 µM of each dNTP, 0.5 U of Ex Taq DNA polymerase, 1X Ex Taq Buffer, and final concentrations of each primer at 0.24 µM. PCR conditions consisted of an initial denaturation at 94 °C for 5 minutes followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 10 minutes (Kim et al., 2015). The isolates identified in the 689 bp were identified as *Vibrio* spp.

*Species-specific multiplex PCR analysis*

Multiplex PCR was performed for species identification of the strains identified as *Vibrio* spp. For the multiplex PCR analysis used for the detection of *Vibrio* species, a total volume of 25 µl was prepared containing 25 ng of sample DNA, 200 µM of each dNTP, 0.5 U of Ex Taq DNA polymerase, 1X Ex Taq Buffer, and primer concentrations as specified in Table 1. PCR conditions consisted of an initial denaturation at 94°C for 5 minutes followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes (Kim et al., 2014; 2015). Following the 2% agarose gel electrophoresis, the agarose gel was analysed using a gel documentation system (Vilber Lourmat®, France) and the presence of bands at 297 bp for *Vibrio parahaemolyticus*, 154 bp for *Vibrio cholerae*, 484 bp for *Vibrio vulnificus*, 199 bp for *Vibrio alginolyticus*, 249 bp for *Vibrio mimicus*, and 601 bp for *Vibrio harveyi* were identified (Kim et al., 2014; 2015).

*Antibiotic Susceptibility Tests of Vibrio Isolates*

The antibiotic susceptibility of *Vibrio* strains was examined using the Kirby Bauer Disk diffusion method. For this purpose, antibiotic disks containing tetracycline (30 µg/disk), streptomycin (10 µg/disk), sulfamethoxazole-trimethoprim (25 µg/disk), gentamicin (10 µg/disk), enrof-

**Table 2.** List of primers used to identify antibiotic resistance genes

| Primer name | Primer Sequence (5'-3') | Amplicon size (bp) | Target Gene |
|---|---|---|---|
| *aadA1-F*<br>*aadA1-R* | TATCCAGCTAAGCGCGAACT<br>ATTTGCCGACTACCTTGGTC | 447 | Streptomycin resistance |
| *tetA-F*<br>*tetA-R* | GGTTCACTCGAACGACGTCA<br>CTGTCCGACAAGTTGCATGA | 577 | Tetracycline resistance |
| *tetB-F*<br>*tetB-R* | CCTCAGCTTCTCAACGCGTG<br>GCACCTTGCTGATGACTCTT | 634 | Tetracycline resistance |
| *dfrA1-F*<br>*dfrA1-R* | GGAGTGCCAAAGGTGAACAGC<br>GAGGCGAAGTCTTGGGTAAAAAC | 367 | Trimethoprim resistance |
| *Qnr-F*<br>*Qnr-R* | GGGTATGGATATTATTGATAAAG<br>CTAATCCGGCAGCACTATTTA | 670 | Fluoroquinolone resistance |
| *aac[3]-IV-F*<br>*aac[3]-IV-R* | CTTCAGGATGGCAAGTTGGT<br>TCATCTCGTTCTCCGCTCAT | 286 | Gentamicin resistance |
| *Sul1-F*<br>*Sul1-R* | TTCGGCATTCTGAATCTCAC<br>ATGATCTAACCCTCGGTCTC | 822 | Sullphonamide resistance |
| *blaSHV-F*<br>*blaSHV-R* | TCGCCTGTGTATTATCTCCC<br>CGCAGATAAATCACCACAATG | 768 | Cephalothin resistance |
| *CITM-F*<br>*CITM-R* | TGGCCAGAACTGACAGGCAAA<br>TTTCTCCTGAACGTGGCTGGC | 462 | Ampicillin resistance |
| *ereA-F*<br>*ereA-R* | GCCGGTGCTCATGAACTTGAG<br>CGACTCTATTCGATCAGAGGC | 419 | Erythromycin resistance |

loxacin (5 µg/disk), cefalothin (30 µg/disk), ampicillin (10 µg/disk), and florfenicol (30 µg/disk) (Oxoid, UK) were used. After incubation, the inhibition zone diameters around the disks were measured, and the susceptibility or resistance of the respective isolate to antibiotics was evaluated according to CLSI standards (CLSI, 2018).

*Multiplex PCR for Determining Antibiotic Resistance Genes*

To determine the antibiotic resistance genes of molecularly typed *Vibrio* strains, multiplex PCR was performed using the primers specified in Table 2 (Shahrani et al., 2014). PCR amplification was carried out in a 25 µl total volume, containing 5 µl of 10X Taq enzyme buffer solution, 25 mM magnesium chloride ($MgCl_2$), 200 µmol of each dNTP, 2 U Taq DNA polymerase, and 3 µl template DNA. The concentrations of each primer in the prepared master mixes were adjusted to 0.5 µM. PCR conditions included an initial denaturation at 94°C for 8 minutes, followed by 32 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 70 seconds, extension at 72°C for 2 minutes, and a final extension at 7 °C for 8 minutes. PCR products were run on a 1.5% agarose gel to visualize bands of the sizes specified in Table 2 using a gel documentation system.

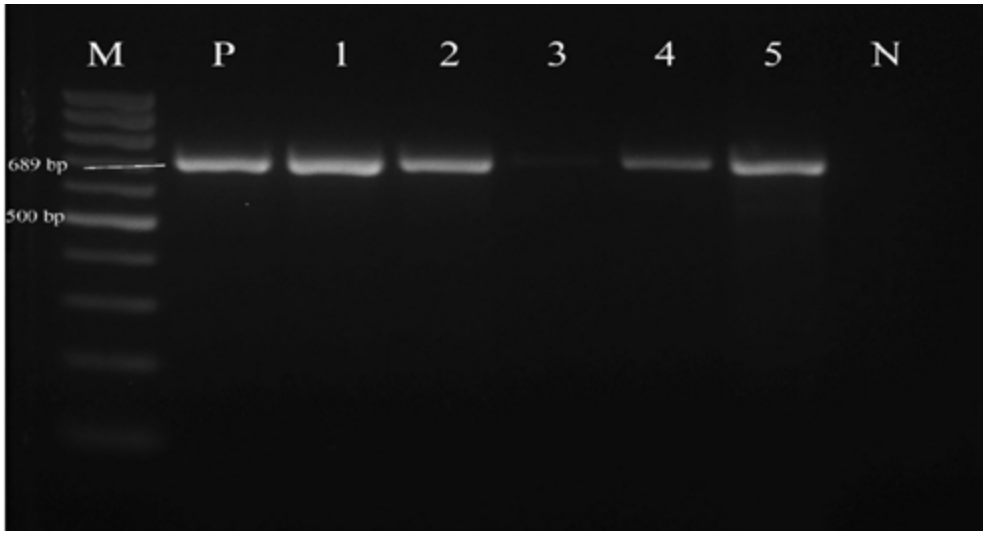


**Figure 1.** 16S rRNA PCR analysis electrophoresis image of *Vibrio* species. M: Molecular marker 100 bp; P: *Vibrio alginolyticus* ATCC 17749 positive control; N: Negative control (sterilised $ddH_2O$); 1-5: *Vibrio* spp. positive samples

## Results

*Phenotypic Isolation and Identification Results for Vibrio spp.*

In this study, bacterial growth was observed in 46 out of 100 samples (46%). The suspected colonies were determined to be round, smooth, translucent, and cream-colored and were subjected to Gram staining. The Gram staining results showed that the 46 *Vibrio*-suspected colonies were Gram-negative and comma-shaped bacteria and were positive for both catalase and oxidase tests. These colonies were sensitive to the Vibriostatic agent O129. The 46 suspected *Vibrio* colonies were then subcultured onto TCBS agar, where 25 (54.3%) of the colonies formed yellow colonies and 21 (45.6%) formed green colonies. The isolates confirmed by growth on the Vibrio selective medium TCBS were identified as *Vibrio* spp.

*Phenotypic Identification Results of Vibrio spp. Using BD Phoenix™*

Among the 46 isolates identified as *Vibrio* spp. based on their growth on *Vibrio* selective TCBS agar, identification using the BD Phoenix™ M50 system revealed that 43 (93.5%) of the isolates were *Vibrio alginolyticus*, while 3 (6.5%) were *Vibrio parahaemolyticus*.

*Genotypic Identification Results*

*16S rRNA PCR results*

16S rRNA PCR analysis was conducted on the 46 *Vibrio* spp. isolates identified by phenotypic methods. During electrophoresis, samples within the range of 689 bp were examined, and all 46 strains (100%) were identified as *Vibrio* spp. (Figure 1).

*Multiplex PCR analysis results*

Multiplex PCR was performed to identify the species of the 46 isolates determined to be *Vibrio* spp. The multiplex PCR results showed bands in the 199 bp range in 22 isolates (48%), identifying them as *V. alginolyticus*; bands in the 601 bp range in 13 isolates (28%), identifying them as *V. harveyi*; bands in the 297 bp range in 3 isolates (7%), identifying them as *V. parahaemolyticus*; and a band at 484 bp in 1 isolate (2%), identifying it as *V. vulnificus*. The analysis did not show band formation at 249 bp and 154 bp, indicating that there were no isolates of *V. mimicus* and *V. cholerae* (Figure 2). In the remaining 7 isolates (15%), no bands were observed from the multiplex PCR, and these isolates were determined to be *Vibrio* spp. (Figure 3).

*Antibiogram Results*

An antibiogram was performed on 46 identified *Vibrio* isolates using the Kirby-Bauer disk diffusion method. The results of the antibiogram are shown in Table 3. The *Vibrio* isolates (n=46) were resistant to 84.8% of the ampicillin and 54.4% of the streptomycin.

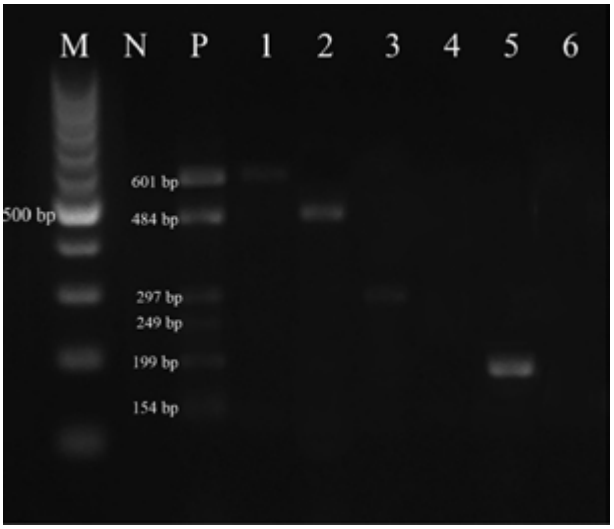


**Figure 2.** Multiplex PCR analysis electrophoresis image of *Vibrio* species. M: Molecular marker 100 bp; P: *Vibrio* positive control- *Vibrio alginolyticus* ATCC 17749 (199 bp), *Vibrio cholerae* ATCC 39050 (154 bp), *Vibrio harveyi* ATCC 33842 (601 bp), *Vibrio mimicus* ATCC 33653 (249 bp), *Vibrio parahaemolyticus* ATCC 17802 (297 bp), *Vibrio vulnificus* ATCC 27562 (484 bp); N: Negative control; 1: *V. harveyi* positive sample; 2: *V. vulnificus* positive sample; 3: *V. parahaemolyticus* positive sample; 4: *Vibrio* negatives ample; 5: *V. alginolyticus* positive sample; 6: *Vibrio* negative sample.

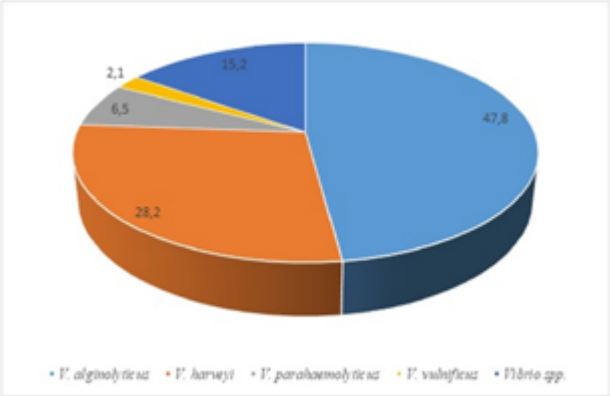


**Figure 3.** Identification percentages of *Vibrio* species

The isolates were found to be 100% susceptible to sulfamethoxazole-trimethoprim and enrofloxacin, 97.8% to florfenicol, 93.5% to tetracycline, 82.6% to cephalothin, and 80.5% to gentamicin. The susceptibility/resistance profiles of the *Vibrio* isolates (n=46) to different antibiotic types are shown in Figure 4.

*Antibiotic Resistance Genes Results*

The results of multiplex PCR analysis performed to detect antibiotic resistance genes of 46 *Vibrio* isolates are shown in Table 4.

Multiplex PCR analysis revealed that trimethoprim resistance gene (*dfrA1*) was detected in 29 (63%) isolates, erythromycin resistance gene (*ereA*) in 27 (58.7%) isolates, gentamicin resistance gene (*aac3-IV*) in 24 (52.2%) isolates, cephalothin resistance gene (*blaSHV*) in 20 (43.5%) isolates, tetracycline resistance genes (8 isolates *tetA*, 3 isolates *tetB*) in 11 (24%) isolates, ampicillin resistance

**Table 3.** Antibiogram results of *Vibrio* isolates

| Sample No | Multiplex PCR | SXT | AMP | KF | ENR | FFC | S* | CN | T |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *V. harveyi* | S | R | S | S | S | R | R | S |
| 2 | *V. alginolyticus* | S | R | S | S | S | R | I | S |
| 3 | *V. harveyi* | S | R | I | S | S | R | I | S |
| 4 | *V. parahaemolyticus* | S | R | S | S | S | R | S | S |
| 5 | *V. alginolyticus* | S | R | S | S | S | R | S | S |
| 6 | *V. alginolyticus* | S | R | S | S | S | I | S | S |
| 7 | *V. alginolyticus* | S | R | S | S | S | R | S | S |
| 8 | *V. alginolyticus* | S | R | S | S | S | I | S | S |
| 9 | *V. alginolyticus* | S | R | S | S | S | I | S | S |
| 10 | *V. alginolyticus* | S | R | S | S | S | R | S | I |
| 11 | *V. alginolyticus* | S | R | S | S | S | R | S | S |
| 12 | *V. alginolyticus* | S | R | S | S | S | I | S | S |
| 13 | *V. alginolyticus* | S | R | S | S | S | R | S | S |
| 14 | *V. harveyi* | S | R | S | S | S | I | S | S |
| 15 | *V. alginolyticus* | S | I | S | S | S | R | S | S |
| 16 | *V. harveyi* | S | R | S | S | S | I | S | S |
| 17 | *V. alginolyticus* | S | R | S | S | S | R | S | S |
| 18 | *V. vulnificus* | S | R | I | S | S | I | I | S |
| 19 | *V. alginolyticus* | S | I | S | S | S | I | R | S |
| 20 | *V. harveyi* | S | R | S | S | S | I | S | S |
| 21 | *V. alginolyticus* | S | R | S | S | S | I | S | S |
| 22 | *V. harveyi* | S | R | I | S | S | R | S | S |
| 23 | *V. alginolyticus* | S | R | S | S | S | I | S | S |
| 24 | *V. harveyi* | S | R | R | S | S | I | S | S |
| 25 | *V. harveyi* | S | R | I | S | S | I | S | S |
| 26 | *Vibrio* spp. | S | R | S | S | S | R | S | S |
| 27 | *V. alginolyticus* | S | R | S | S | S | R | S | S |
| 28 | *V. alginolyticus* | S | R | S | S | S | R | S | S |
| 29 | *V. alginolyticus* | S | I | S | S | S | R | S | R |
| 30 | *V. alginolyticus* | S | I | S | S | S | I | S | S |
| 31 | *Vibrio* spp. | S | R | S | S | I | R | S | S |
| 32 | *V. alginolyticus* | S | R | S | S | S | I | S | S |
| 33 | *V. alginolyticus* | S | R | S | S | S | R | I | S |
| 34 | *Vibrio* spp. | S | S | S | S | S | R | S | S |
| 35 | *Vibrio* spp. | S | R | S | S | S | I | S | S |
| 36 | *V. parahaemolyticus* | S | S | S | S | S | S | S | S |
| 37 | *V. harveyi* | S | R | S | S | S | I | S | S |
| 38 | *V. parahaemolyticus* | S | S | S | S | S | R | S | S |
| 39 | *V. alginolyticus* | S | R | S | S | S | I | S | S |
| 40 | *Vibrio* spp. | S | R | S | S | S | S | S | S |
| 41 | *V. harveyi* | S | R | S | S | S | R | S | S |
| 42 | *V. harveyi* | S | R | I | S | S | I | I | S |
| 43 | *Vibrio* spp. | S | R | S | S | S | R | I | S |
| 44 | *V. harveyi* | S | R | S | S | S | R | S | S |
| 45 | *V. harveyi* | S | R | R | S | S | R | R | S |
| 46 | *Vibrio* spp. | S | R | R | S | S | R | S | R |

SXT: Sulfamethoxazole-trimethoprim; AMP: Ampicillin; KF: Cephalothin; ENR: Enrofloxacin; FFC: Florfenicol; S*: Streptomycin; CN: Gentamicin; T: Tetracycline; S: Susceptible; I: Intermediate; R: Resistance

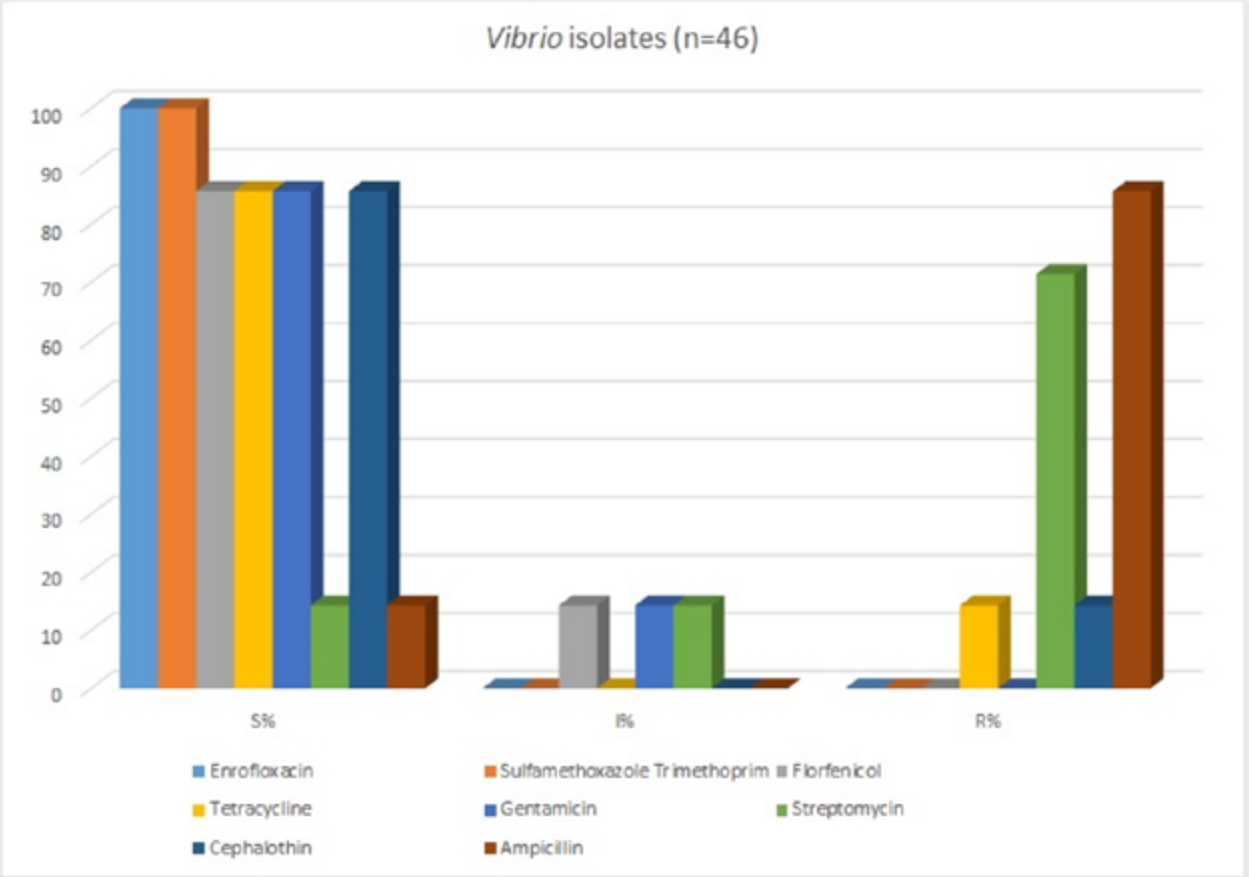


**Figure 4.** Antibiotic sensitivity/resistance percentages of *Vibrio* isolates

gene (*CITM*) in 6 (13%) isolates, sulfonamide resistance gene (*sul1*) in 5 (10.9%) isolates, streptomycin resistance gene (*aadA1*) in 5 (10.9%) isolates, and fluoroquinolone resistance gene (*Qnr*) in 3 (6.5%) isolates, either individually or in multiple combinations. The number of resistance genes present in the *Vibrio* isolates (n=46) was examined, revealing that 2 isolates (4.3%) had 1 resistance gene, 15 isolates (32.6%) had 2 resistance genes, 16 isolates (34.7%) had 3 resistance genes, 6 isolates (13%) had 4 resistance genes, 4 isolates (8.6%) had 5 resistance genes, and 1 isolate (2%) had 7 resistance genes, while 2 isolates (4.3%) did not have any resistance genes.

## Discussion

The aquaculture industry is currently the fastest-growing sector in meeting nutritional standards and contributing to global economic development by providing high protein sources. Bacterial diseases in aquaculture affect both economical and social development in many countries. Vibriosis is one of the most common bacterial diseases affecting various marine fish. The most common species infecting animals in aquaculture farms are *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, *V. owensii*, and *V. campbellii* (Ina-Salwany et al., 2019).

Our study comprised 100 suspected sea bass with vibriosis. Through bacteriological culturing, biochemical analyses, and TCBS agar incubation, 46 isolates (46%) were isolated as *Vibrio* spp. It was determined that 25 colonies (54.4%) on TCBS agar were yellow, while 21 colonies (45.6%) were green. For species-level identification, the BD Phoenix automated identification system was utilized, identifying 43 isolates (93.5%) as *V. alginolyticus* and 3 isolates (6.5%) as *V. parahaemolyticus*. All isolates (n=46) were confirmed as *Vibrio* spp. using 16S rRNA PCR analysis. Subsequent multiplex PCR analysis for species identification revealed that 22 isolates (48%) were identified as *V. alginolyticus*, 13 isolates (28%) as *V. harveyi*, 3 isolates (7%) as *V. parahaemolyticus*, and 1 isolate (2%) as *V. vulnificus*. Seven isolates (15%) showed no bands in multiplex PCR and were identified as *Vibrio* spp. It was determined in our study that *V. alginolyticus* was the predominant species, as *V. cholerae* and *V. mimicus* species were not isolated and identified from the analysed samples.

In our study, isolation, and identification were initially conducted using conventional methods and the Vibrio selective medium TCBS agar, followed by PCR analyses for species identification. Similar to our research, studies by Raissy et al. (2012), Uzun and Ogut (2015), Suresh et al. (2018), Abd El Tawab et al. (2018), Deng et al. (2020), El-Gamal and El Bahi (2020), Gxalo et al. (2021), Sadat et al. (2021), and Zin et al. (2021) performed *Vibrio* spp. identification primarily using conventional methods and culture on TCBS agar, followed by PCR analyses for species identification.

Our findings from both biochemical analysis-based identification using the BD Phoenix automated identification

**Table 4.** Antibiotic resistance genes detected in *Vibrio* isolates

| Sample No | Multiplex PCR | Antibiotic Resistance Genes | | | | | |
|---|---|---|---|---|---|---|---|
| 1 | *V. harveyi* | *ereA* | *CITM* | *tetB* | *blaSHV* | *sul1* | |
| 2 | *V. alginolyticus* | *aac 3-IV* | *dfrA1* | | | | |
| 3 | *V. harveyi* | *ereA* | *dfrA1* | *tetA* | | | |
| 4 | *V. parahaemolyticus* | *aac 3-IV* | *ereA* | *dfrA1* | *aadA1* | *CITM* | *blaSHV* |
| 5 | *V. alginolyticus* | *aac 3-IV* | *ereA* | *blaSHV* | *sul1* | | |
| 6 | *V. alginolyticus* | *ereA* | *dfrA1* | | | | |
| 7 | *V. alginolyticus* | *ereA* | *dfrA1* | | | | |
| 8 | *V. alginolyticus* | *aac 3-IV* | *blaSHV* | *sul1* | | | |
| 9 | *V. alginolyticus* | *aac 3-IV* | *dfrA1* | | | | |
| 10 | *V. alginolyticus* | *aac 3-IV* | *ereA* | *dfrA1* | *blaSHV* | | |
| 11 | *V. alginolyticus* | *aac 3-IV* | *ereA* | *dfrA1* | *blaSHV* | | |
| 12 | *V. alginolyticus* | *ereA* | *dfrA1* | | | | |
| 13 | *V. alginolyticus* | *aac 3-IV* | *dfrA1* | *ereA* | *blaSHV* | | |
| 14 | *V. harveyi* | *ereA* | *dfrA1* | *blaSHV* | | | |
| 15 | *V. alginolyticus* | *aac 3-IV* | *ereA* | | | | |
| 16 | *V. harveyi* | *ereA* | *dfrA1* | | | | |
| 17 | *V. alginolyticus* | *aac 3-IV* | *blaSHV* | | | | |
| 18 | *V. vulnificus* | *dfrA1* | *CITM* | *tetA* | *Qnr* | *sul1* | |
| 19 | *V. alginolyticus* | | | | | | |
| 20 | *V. harveyi* | *aadA1* | *Qnr* | | | | |
| 21 | *V. alginolyticus* | *aac 3-IV* | *ereA* | *blaSHV* | | | |
| 22 | *V. harveyi* | *ereA* | *aadA1* | *tetA* | *blaSHV* | | |
| 23 | *V. alginolyticus* | *dfrA1* | *aadA1* | | | | |
| 24 | *V. harveyi* | *ereA* | *dfrA1* | *tetA* | | | |
| 25 | *V. harveyi* | *aac 3-IV* | *dfrA1* | *tetA* | | | |
| 26 | *Vibrio* spp. | *aac 3-IV* | *ereA* | *dfrA1* | *aadA1* | *blaSHV* | |
| 27 | *V. alginolyticus* | *aac 3-IV* | *ereA* | *blaSHV* | | | |
| 28 | *V. alginolyticus* | *aac 3-IV* | *ereA* | *blaSHV* | | | |
| 29 | *V. alginolyticus* | *aac 3-IV* | *blaSHV* | | | | |
| 30 | *V. alginolyticus* | *aac 3-IV* | | | | | |
| 31 | *Vibrio* spp. | *ereA* | *dfrA1* | *sul1* | | | |
| 32 | *V. alginolyticus* | *aac 3-IV* | *ereA* | *blaSHV* | | | |
| 33 | *V. alginolyticus* | *aac 3-IV* | *ereA* | *blaSHV* | | | |
| 34 | *Vibrio* spp. | *aac 3-IV* | *ereA* | *CITM* | | | |
| 35 | *Vibrio* spp. | *aac 3-IV* | *ereA* | *dfrA1* | *tetA* | *blaSHV* | |
| 36 | *V. parahaemolyticus* | *aac 3-IV* | *dfrA1* | *CITM* | *blaSHV* | | |
| 37 | *V. harveyi* | *ereA* | *dfrA1* | *blaSHV* | | | |
| 38 | *V. parahaemolyticus* | *ereA* | *dfrA1* | *tetB* | | | |
| 39 | *V. alginolyticus* | | | | | | |
| 40 | *Vibrio* spp. | *aac 3-IV* | *dfrA1* | | | | |
| 41 | *V. harveyi* | *ereA* | *dfrA1* | *Qnr* | | | |
| 42 | *V. harveyi* | *dfrA1* | *tetB* | | | | |
| 43 | *Vibrio* spp. | *dfrA1* | | | | | |
| 44 | *V. harveyi* | *aac 3-IV* | *dfrA1* | *tetA* | | | |
| 45 | *V. harveyi* | *dfrA1* | *tetA* | | | | |
| 46 | *Vibrio* spp. | *dfrA1* | *CITM* | | | | |

52 colonies were obtained on TCBS media, 34 of which were yellow, and 18 were green. The 18 (34.6%) green colonies were identified as *V. parahaemolyticus*. API 20E tests identified 15 isolates as *V. parahaemolyticus*, and sequencing confirmed 18 isolates as *V. parahaemolyticus*. Comparing our study with these studies reveals differences in identification methods, species diversity investigated, and water temperature parameters, which could explain the discrepancies in our findings.

Duman et al. (2023) investigated the genetic relationship of Vibrio species isolated from fish farms in Türkiye. For this purpose, a total of 256 Vibrio isolates were typed using multilocus sequence analysis (MLSA) and typing (MLST) methods. For all isolates studied (n = 256), diversity analysis, population structure, determination of recombination, demographic history and gene flow were performed according to the MLST scheme. As a result of the study, Vibrio isolates showed high diversity within the Vibrio population and also genetic interactions within the genus. 17 new sequence types were identified by MLST analysis isolated from rainbow trout, sea bream and sea bass in fish farms in Türkiye, and it was emphasized by the researchers that this situation clearly showed that genes frequently undergo recombination. As a result of the research, it was concluded that *V. anguillarum* and *V. alginolyticus* are the dominant species fish farms in Türkiye and have biochemically heterogeneous properties, but it is argued that genotype differences should be evaluated in case of a disease or to prevent measurements.

In our study, *Vibrio* isolates were resistant to ampicillin at a rate of 84.8%, streptomycin at 54.4%, cephalothin at 6.5%, gentamicin at 6.5%, and tetracycline at 4.3%. The isolates were found to be 100% sensitive to sulfamethoxazole-trimethoprim and enrofloxacin, 97.8% sensitive to florfenicol, 93.5% sensitive to tetracycline, 82.6% sensitive to cephalothin, and 80.5% sensitive to gentamicin. The highest resistance was observed against ampicillin. Raissy et al. (2012) performed a disk diffusion analysis to determine the antibiotic resistance profiles of *Vibrio* spp. isolates (n=72) and found that 70 (97.2%) isolates were resistant to ampicillin. Similarly, Suresh et al. (2018) reported that 93.38% of their 15 *Vibrio* spp. isolates were resistant to ampicillin, with the highest resistance observed against this antibiotic. Our study also found that the highest resistance among all *Vibrio* isolates was to ampicillin (84.8%), consistent with these studies.

In contrast to our findings, Raissy et al. (2012) reported that of the 72 *Vibrio* spp. isolates, 60 (83.3%) were resistant to gentamicin, 18 (25%) to streptomycin, and 13 (18.1%) to tetracycline. Suresh et al. (2018) observed that their Vibrio spp. isolates exhibited high levels of resistance to gentamicin (80%), tetracycline (33.33%), and streptomycin (6.66%). Deng et al. (2020) used the disk diffusion method to for the antibiogram of 70 *Vibrio* spp. isolates and found resistance rates of 12.8% to gentamicin, 10% to tetracycline, and 7.1% to sulfamethoxazole-trimethoprim. The differences in findings between our study and these studies are thought to be due to variations in the antibiotics used for treatment.

In our antibiogram results, it was determined that 21 isolates (45.6%) were resistant to 1 antibiotic type, 19 isolates (41.3%) to 2 antibiotic types, 1 isolate (2%) to 3 antibiotic types, and 2 isolates (4%) to 4 antibiotic types. Evaluation by antibiotic groups revealed that 44 isolates (95%) were resistant to at least 1 antibiotic group, 20 isolates (43.4%) to 2 antibiotic groups, and 2 isolates (4.3%) to 3 antibiotic groups. Two isolates (4.6%) did not develop resistance to any antibiotic group. Shahimi et al. (2021) found that 45.8% of *V. alginolyticus* isolates were resistant to one or more antibiotics; Raissy et al. (2012) found that 11% of *Vibrio* isolates were resistant to 4 antibiotics, 26.4% to 5 antibiotics, 13.8% to 6 antibiotics, 47.2% to 7 antibiotics, and 1.3% to 8 antibiotics; Yang et al. (2017) reported that 68.38% of *Vibrio* isolates were resistant to more than 3 antibiotics. It is noteworthy that the resistance rates and resistance diversity obtained in these studies are higher than the research findings we obtained. This difference may be attributed to variations in treatment protocols and the diversity of antibiotics used in the regions where the studies were conducted, in comparison to the findings of our study.

When we examined the number of resistance genes in our study, 1 resistance gene in 2 (4.3%) isolates, 2 resistance genes in 15 (32.6%) isolates, 3 resistance genes in 16 (34.7%) isolates, 4 resistance genes in 6 (13%) isolates, 5 resistance genes in 4 (8.6%) isolates, 7 resistance genes in 1 (2%) isolate were determined. It was observed that no resistance gene was present in two isolates (4.3%). Trimethoprim resistance gene in 29 (63%) isolates, erythromycin resistance gene in 27 (58%) isolates, gentamicin resistance gene in 24 (52%) isolates, cephalothin resistance gene in 20 (43%) isolates, tetracycline gene in 11 (24%) isolates, ampicillin resistance gene in 6 (13%) isolates, sulphonamide resistance gene in 5 (11%) isolates, streptomycin resistance gene in 5 (11%) isolates, and fluoroquinolone resistance gene in 3 (7%) isolates were detected with single or multiple combinations. Raissy et al. (2012) reported that streptomycin resistance gene was detected in 18 (25%) of *Vibrio* isolates (n = 72), tetracycline resistance gene was detected in 6 (8.3%), and erythromycin resistance gene was detected in 5 (7%). Gxalo et al. (2021) stated that in the PCR analysis performed for the presence of the resistance gene, the ampicillin resistance gene was detected in all (100%) *V. vulnificus* isolates. Faja et al. (2019) stated that the highest resistance gene (80.64%) belongs to beta-lactamases, and the lowest resistance gene (16.12%) belongs to florfenicol. When these studies are examined, antibiotic resistance genes also show differences in isolates with multiple antibiotic resistance. In our research, the highest antibiotic resistance gene detected in *Vibrio* isolates was trimethoprim, and the lowest resistance gene was the fluoroquinolone resistance gene.

## Conclusion

Aquaculture has an important place in the supply of animal protein in the world and in our country. In addition to its contributions to the food chain, it also contributes to the country's economy by providing employment and exports. The healthy maintenance of the created system is possible with fish health. Diseases damage the economy of businesses by increasing workload, increasing antibiotic costs, and failing to achieve anticipated sales. It is known that *Vibrio* species are frequently encountered in aquaculture and are one of the primary factors that cause deaths.

Appropriate determination of the technique chosen for the diagnosis of diseases and the chosen treatment method is essential to preventing losses. As a result of incorrect identification and antibiotic sensitivity tests, microorganisms gain resistance, and the use of antibiotics to which microorganisms are not sensitive causes both the prolongation of the treatment process, unnecessary antibiotic costs, and antibiotic residues in the seas and aquaculture systems.

In our research, *Vibrio* species in dead sea bass fish with suspicion of vibriosis were investigated. As a result of the analyses, the presence of *Vibrio* species in sea bass fish was detected molecularly. As a result of PCR analyses of antibiogram and antibiotic resistance genes, it was revealed that multiple antibiotic resistances developed in the *Vibrio* species obtained. It is recommended that the identification of *Vibrio* species be done with molecular methods instead of conventional methods, and that antibiotic species that are sensitive as a result of antibiotic sensitivity or resistance tests be used in antibiotic selection.

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## Author contribution statement

Concept: O.D., S.K., H.T.Y.D.; Design: O.D., S.K., H.T.Y.D.; Data Collection or Processing: O.D., S.K., H.T.Y.D.; Analysis or Interpretation: O.D., H.T.Y.D.; Literature Search: O.D., S.K., H.T.Y.D.; Writing: O.D., S.K., H.T.Y.D.

## Conflict of interest

The authors declare that they have no conflict of interest in this study.

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