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Investigation for the presence of bacteria and antimicrobial resistance genes in sea snails (*Rapana venosa*)

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Abstract

Introduction and Objective. The aims of this study were to search for the presence of bacteria in sea snails (*Rapana venosa*) by using culturomics and Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and the antibiotic resistance/susceptibility of the sea snails.

Materials and method. The anti-microbial susceptibilities of Gram-negative bacteriawas assessed by the Kirby-Bauer disk diffusion method, the presence of the *mcr* genes (*mcr*-1 to -5), the major carbapenemase and β-lactamase resistant genes in Gram-negative bacteria, using mPCR method and 16S rRNA sequence analysis of *A. hydrophila* isolates.

Results. Bacterial growth accounted for 100% and 94.2% in the samples of intestine and meat, respectively, in the snails. The main organisms identified by MALDI-TOF MS were *A. salmonicida* subsp. *salmonicida* at 33.7%, followed by *Raoultella ornithinolytica* at 9.6% (10/104) and *Staphylococcus warneri* at 7.7% in meat and intestine samples. *Aeromonas hydrophila/punctata (caviae), Aeromonas sobria, Klebsiella aerogenes, Klebsiella oxytoca, Raoultella planticola, Shewanella putrefaciens* and *Vibrio vulnificus* are intrinsic or chromosomally-mediated resistant against ampicillin. No *mcr* genes (*mcr*-1 to -5), the major carbapenemase and β-lactamase resistant genes were found. *Aeromonas salmonicida* subsp. *salmonicida* showed very low levofloxacin and meropenem resistance levels at 2.9%. When the sequence was searched in the Blast database, the genome of *A. hydrophila/punctata (caviae)* isolate showed high similarity with the *A. hydrophila* sequences.

Conclusions. The findings obtained not only provide data about the proportion of bacteria in the gut and meat of the sea snails and their antibiotic resistance/susceptibility, but also show the absence of carbapenemase, colistin, and β-lactamase resistant genes among bacterial isolates from sea snail gut microbes.

Key words

Carbapenem, colistin, MALDI-TOF MS, resistance genes, sea snail

INTRODUCTION

Snail production and marketing, although very important for the economy of many countries, is often neglected, specifically in developed countries [1]. Sea snails generally feed on aquatic creatures, such as mussels, oysters, small crustaceans and other bivalves, and it has been reported that the *Rapana venosa* species of sea snails, which grows up to 20 cm in length in the Black Sea [2]. They mostly live on rocky, sandy, and muddy bottoms. Sea snails are among the sea products that are not consumed in Turkey but are of great importance economically due to their export to Japan, China, and some European countries. Some of them are consumed in touristic areas of Turkey [3].

Sea snails were reported to harbor various pathogenic bacteria, e.g., *E. coli*, coliforms, and *Enterococcus* spp, causing diseases in humans if they are not appropriately processed before consumption [4]. In a study performed to analyze the bacterial flora compound in the esophagus and intestinal tract of wild *Rapana venosa* (sea snail) by 16S rRNA gene sequencing, the 10 most commonly identified microbial genera in the esophagu were *Bifidobacterium*, *Escherichia*, *Enterococcus*, *Mycoplasma*, *Shewanella*, *Vibrio*, *Octadecabacter*, *Lactobacillus*, *Streptococcus*, and *Sphingomonas*, which represented for 41.35% of total microbes [5]. In addition, the same study indicated that the most abundant microbial genera in the intestine were *Bifidobacterium, Escherichia, Mycoplasma, Psychrilyobacter, Peptoniphilus, Shewanella, Sporobacterium, Vibrio, Octadecabacter,* and *Mobiluncusin,* which accounted for 62.74% of microbes [5].

In recent years, colistin has been contemplated as one of the last-resort antibiotics to treat increasingly complex human infections [6, 7]. Mobilized colistin resistance gene (*mcr*-1), which is a plasmid-mediated colistin resistance gene in Enterobacterales, was the first reported gene in *Escherichia coli* (*E. coli*) isolated in China in 2016 [8]. Later, *mcr*-2 [9], *mcr*-3 [10], *mcr*-4 [11], *mcr*-5 [12], *mcr*-6 [13], *mcr*-7 [14],

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mcr-8 [15], *mcr*-9 [16] and *mcr*-10 [17] were identified in *Enterobacteriales*.

Since 2010, an increase in *mcr* positive isolates has been observed in both humans and animals [18]. While all *mcr* genes (*mcr*-1 to 10) have been identified in animal samples, *mcr*-6 and *mcr*-7 have not been isolated from human samples [19]. The *mcr* has been the most commonly observed gene in *E. coli*, but is also available in different genera including Escherichia, Enterobacter, Klebsiella, Salmonella, and Shigella [19].

Carbapenems are β-lactams and are the most effective antibiotics against Gram-negative bacterial infections [20]. Carbapenem-resistant *Enterobacteriaceae* (CRE) have shown rapid growth and are found worldwide [21]. Carbapenemases (carbapenem-hydrolyzing β-lactamases) are divided into molecular classes A, B, and D [22]. Oxacillin-hydrolyzing (OXA)-48, a class D carbapenemase, is of great concern due to its difficulty in detection and high chances of escape from the treatment [21].

The extended-spectrum β-lactamase (ESBL) producing enterobacterial strains are a major threat to public health because of their extended cephalosporin and monobactam resistance [23]. ESBL-producing *Escherichia coli* (*E. coli)* has been detected in milk, cattle meat, and fish in Asia, Europe, and other parts of the world [24]. The ESBL-producing *E. coli* are resistant to penicillin, cephalosporin, and aztreonam by virtue of cefotaxime-hydrolyzing β-lactamase–Munich (CTX-M), temoneira class A extended-spectrum β-lactamase (TEM) and sulfhydryl variant of the TEM enzyme (SHV) βlactamases. Among these types, over the last decades, CTX-M-enzymes are the most predominant ESBL in humans and animals worldwide [25] and are known to be an increasingly serious public health concern [26].

Although the source of the *mcr*-1-positive isolates in sea snails remains unknown, the aquatic systems, particularly the aquaculture industry, can be an important reservoir for colistin resistance genes [27]. A few studies have identified *E. coli* positive for the *mcr-1* gene and *Aeromonas* positive for the *mcr*-3 gene from aquaculture [27]. Recently, Shen et al. [28] reported a strong association between aquaculture, aquatic food products, and *mcr-1*-positive *E. coli* in human intestinal samples. Although only two studies investigating bacteria in sea snails have been performed in Turkey [29, 30], there is no data available about the prevalence of colistin and carbapenem resistance genes in sea snails in Turkey. In many *mcr* gene studies where plasmid-derived colistin resistance has been determined, the *mcr* gene has been detected at different rates in humans and animals. However, no studies have been reported on the *mcr* gene in snails. Therefore, it is important to investigate carbapenem (bla_{NDM-}1, bla_{OXA-48}, bla_{IMP}, bla_{VIM}, and bla_{KPC}) and colistin resistance genes (*mcr*) in the global aquaculture.

Since 2010, identification of the microorganisms in the field of clinical microbiology has been successfully performed by the use of matrix-assisted laser-desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF MS), together with various other molecular and biochemical identification methods. However, MALDI-TOF MS has more advantages, including speed, accuracy, low cost, and ease of operation. Recent developments with database standardization and algorithm optimization have made the use of MALDI-TOF more convenient in the identification of resistant strains [31, 32]. Another advantage of the MALDI-TOF is its ability to identify pathogens directly from clinical samples – urine, blood, and cerebrospinal fluids [33, 34]. With this technique it is very easy to identify gram-positive, gram-negative, aerobic, and anaerobic bacteria, fungi, viruses, yeasts, and mycobacteria directly from the clinical samples. This is also a powerful method for identifying bacteria at the species level.

The MALDI-TOF MS has been used successfully previously to identify both gram-positive and gram-negative bacteria from the clinical samples [35], and the rate of identification of gram-positive bacteria is higher than that of Gram-negative bacteria. However, this process needs a large number of samples [36, 37]. Previously, both gram-positive and gram-negative bacteria were identified by MALDI-TOF MS with 100% concordance with gram-negative bacteria and 96% concordance with gram-positive bacteria [35].

There are certain limitations of MALDI-TOF MS in the identification of pathogens from the clinical samples:

1. Accuracy of identification is low with the pathogens having similar mass spectra, ex: identification of *E. coli* and *Shigella*.
2. Difficulty in differentiating the strains in complex bacterial groups, e.g., *Enterobacter cloacae* complex, *Streptococcus mitis* (*S. mitis*) complex and *M. tuberculosis* complex [38, 39].
3. If there are only a few mass spectra for some species in the database, identification accuracy is low.
4. Enrichment of the spectral database and improvement in the quality is needed.
5. It can not differentiate the closely-related organisms in multiple culture samples, or in samples with less purity.

OBJECTIVE

The aims of the study were to analyze the presence of bacteria in the sea snail samples by using culture and matrix-assisted laser-desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF MS), as well as the anti-microbial susceptibilities of gram-negative bacteria assessed by the Kirby-Bauer disk diffusion method, to detect *mcr* and bla_{NDM}, bla_{KPC}, bla_{VIM-1}, bla_{IMP-1} and bla_{OXA-48} – the most common carbapenemases and β-lactamase (bla_{TEM1}, bla_{CTX-M}, bla_{SHV-1}) resistant genes – by using the multiplex polymerase chain reaction (PCR) method, and determination of the genetic characteristics of *Aeromonas hydrophila* (*A. hydrophila)* isolates by 16S rRNA sequence analysis.

MATERIALS AND METHOD

Samples. The study was carried out according to ethical standards and approved by the Firat University Ethics Committee (Protocol No. FU-2021/34891). In the 2021 fishing season a total of 104 sea snails (*Rapana venosa*) were purchased from commercial fishing boats engaged in sea snail fishing off the coast of Dereköy, Samsun province, on the Black Sea coast of Turkey. Snails were brought to the Fish Ecophysiology Laboratory of the Faculty of Fisheries under cold chain, and the gender of each snail sample determined by the presence or absence of a penis, weighed individually to the nearest 0.01 g using an electronic balance (GF-6100, A&D Company Ltd, Japan). Their total shell length was measured with a digital caliper (INSIZE 1108–300, Germany). The collected sea snails were cleaned with water, the shells were wiped with 70% ethyl

alcohol, and broken with a glass hammer and the intestinal parts removed with sterile forceps by cutting with the help of a scalpel under aseptic conditions. The intestines and meat samples of each snail were collected in separate sterile bags and delivered immediately to the Molecular Microbiology Laboratory at the İnönü University Faculty of Medicine, Turgut Ozal Medical Centre, for microbiological analysis under cold chain.

Bacterial culture and isolation. Twenty-five grams of sea snail meat and one gram of sea snail intestine samples were collected into sterile bags containing 225 ml and 9 ml of buffered peptone water (BPW), respectively, and homogenized in a stomacher device for 2 minutes. For the isolation of Enterobacterales, 10 µL of the homogenate was taken with a plastic loop and plated on MacConkey agar and 5% sheep blood agar media, and subsequently incubated at 37 °C for 18–24 hours under aerobic conditions. All suspicious Enterobacterales colonies were purified and identified by MALDI-TOF MS, following biochemical methods.

For S*almonella* isolation, the Bacteriological Analytical Manual method [40] was used. One ml of the homogenates of meat and intestines of sea snail was transferred from the pre-enrichment medium and placed into 10 mL Rappaport-Vassiliadis (RVS) liquid nutrient and incubated at 42 °C for 18–24 hours. Ten microlitres were taken from the selective enrichment culture with a plastic loop and plated onto Hektoen-Enteric agar and 5% sheep blood agar media. The plates were incubated for 18–24 hours at 37 °C under aerobic conditions [41]. The positive colonies were purified and identified using the MALDI-TOF MS.

MALDI-TOF MS analysis. MALDI-TOF MS analysis was conducted as per described by Dubois *et al.* [42] and Westblade et al. [43]. A schematic diagram showing the work-flow in a MALDI-TOF MS is shown in Figure 1. Before undertaking any identification of the microorganisms, internal QC was performed for automatic instrument calibration using manufacture-specified calibration standards. Each strain of interest was included during the calibration. During calibration, the generated mass spectrum was analyzed to check the baseline of the spectrum, and confirmed the presence of calibration peaks. The calibrated spectrum was run against the reference database ensure the correct identification with a given level of confidence as per the manufacturer's specifications. The calibration was performed before every run and all the calibration results recorded. Briefly, a single colony was selected using a sterile toothpick from the colonies grown on blood agar and transferred onto two separate spots of the MALDI-TOF MS disposable plate. The spots were then coated with 1 µL of 70% formic acid and matrix solution (α-Cyano-4-hydroxycinnamic acid, CHCA), respectively, and allowed to air dry for 1–2 min at room temperature. *E. coli* ATCC 8739 was employed for the calibration process as the reference strain and was utilized on the MALDI-TOF MS disposable plate for each group [42, 43]. After the plate had been placed in the Microflex LT MALDI-TOF MS (bioMerieux, France) device for analysis, each sample was exposed to laser shots at 320–400. Isolates were identified with the '*In Vitro* Diagnostic (IVD)' module V3.0 database using the Vitek MS (Bio Mérieux, France) device. After spectra acquisition, the results were transferred from the Acquisition Station software to the VITEK MS Analysis Server (Myla version 4.4) where the identification results were listed [42, 43]. The following calibration settings for the MALDI-TOF MS were used: VITEK MS V3.0 CLI, Acquisition SW: V1.5.0.4, Pre PC: V2.3.3, Myla: 49.0.

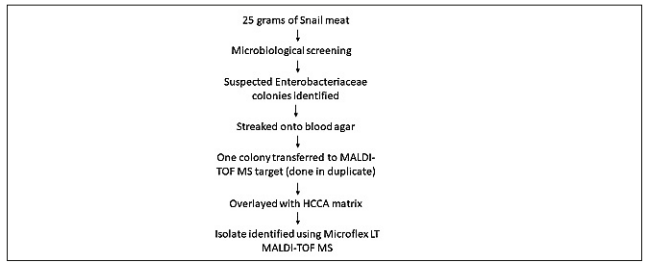

Figure 1. Schematic diagram showing work-flow in a MALDI-TOF MS

Detection of *mcr* and carbapenemase gene using multiplex PCR. DNA extraction was performed on all gram-negative isolates. Genomic DNA was isolated on the QIAsymphony instrument by the QIAsymphony DSP Virus / Pathogen midi kit (Qiagen, Hilden, Germany), as described by the manufacturer's guidelines. It was amplified by multiplex PCR using primers specific to ***mcr***-1–5, a carbapenem (***bla***$_{\text{NDM-1}}$, ***bla***$_{\text{OXA-48}}$, ***bla***$_{\text{IMP}}$, ***bla***$_{\text{VIM,}}$ and ***bla***$_{\text{KPC}}$), and β-lactamase (***bla***$_{\text{TEM-1}}$, ***bla***$_{\text{CTX-M}}$, ***bla***$_{\text{SHV-1}}$) resistance genes (Tab. 1) [44–46].

Table 1. Primers used for *mcr* and other resistance genes

Primers	Primer sequences (5'-3')	Gene region	Amplicon size **(bp)**	References
mcr-1_320bp_fw	AGTCCGTTTGTTCTTGTGGC	*mcr*-1	320	[48]
mcr-1_320bp_rev	AGATCCTTGGTCTCGGCTTG			
mcr-2_700bp_fw	CAAGTGTGTTGGTCGCAGTT	*mcr*-2	715	[48]
mcr-2_700bp_rev	TCTAGCCCGACAAGCATACC			
mcr-3_900bp_fw	AAATAAAAATTGTTCCGCTTATG	*mcr*-3	929	[48]
mcr-3_900bp_rev	AATGGAGATCCCCGTTTTT			
mcr-4_1100bp_fw	TCACTTTCATCACTGCGTTG	*mcr*-4	1116	[48]
mcr-4_1100bp_rev	TTGGTCCATGACTACCAATG			
*MCR5*_fw	ATGCGGTTGTCTGCATTTATC	*mcr*-5	1644	[12]
*MCR5*_rev	TCATTGTGGTTGTCCTTTTCTG			
bla$_{\text{IMP}}$-F	GGAATAGAGTGGCTTAAYTCTC	*bla*$_{\text{IMP}}$	232	[49]
bla$_{\text{IMP}}$-R	GGTTTAAYAAAACAACCACC			
bla$_{\text{VIM}}$-F	GATGGTGTTTGGTCGCATA	*bla*$_{\text{VIM}}$	390	[49]
bla$_{\text{VIM}}$ -R	CGAATGCGCAGCACCAG			
bla$_{\text{OXA-48}}$-F	GCGTGGTTAAGGATGAACAC	*bla*$_{\text{OXA-48}}$	438	[49]
bla$_{\text{OXA-48}}$-R	CATCAAGTTCAACCCAACCG			
bla$_{\text{NDM}^{-}_{1}}$-F	GGTTTGGCGATCTGGTTTTC	*bla*$_{\text{NDM-1}}$	621	[49]
bla$_{\text{NDM}^{-}_{1}}$-R	CGGAATGGCTCATCACGATC			
bla$_{\text{KPC}}$-F	CGTCTAGTTCTGCTGTCTTG	*bla*$_{\text{KPC}}$	798	[49]
bla$_{\text{KPC}}$-R	CTTGTCATCCTTGTTAGGCG			
bla$_{\text{TEM-1}}$-F	TCCGGTCATGAGACAATAACC	*bla*$_{\text{TEM-1}}$	931	[44]
bla$_{\text{TEM-1}}$-R	TTGGTCTGACAGTTACCAATGG			
bla$_{\text{SHV}^{-}_{1}}$-F	TGGTTATGCGTTATATTCGCC	*bla*$_{\text{SHV}}$	968	[44]
bla$_{\text{SHV}^{-}_{1}}$-R	GGTTAGCGTTGCCAGTGCT			
bla$_{\text{CTX-M}}$-F	ATGTGCAGYACCA GTAARGTKATGGC	*bla*$_{\text{CTX-M}}$	93	[45]
bla$_{\text{CTX-M}}$-R	TGGGTRAARTARG TSACCAGAAYCAGCGG			

Amplified products were stained with ethidium bromide following electrophoresis and photographed under ultraviolet light using a Gel Logic 2200 imaging system (Kodak Co., Rochester, NY, USA).

Identification of *A. hydrophila* isolates by 16S rRNA sequence analysis. Isolates identified as *A. hydrophila/ punctata (caviae)* by MALDI-TOF MS analysis were performed by 16S rRNA sequence analysis. For this purpose, the EZ1 automated extraction system (Qiagen, Hilden, Germany) was used for nucleic acid isolation from bacterial isolates. The 834 base pair (bp) region of 16S rRNA was replicated using broad-range eubacterial primers (p8FPL 5'-AGTTTGATCCTGGCTCAG-3' and p806R 5'-GACTACCAGGGTATCTAAT-3' [47].

PCR cycling conditions were as follows: initial denaturation was set at 95 °C for 4 minutes, followed by 35 cycles at 95 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 1 minute, and finally at 72 °C for 7 minutes. Before purification of the amplicon, PCR products were confirmed with 2% agarose gel electrophoresis. Appropriately-sized amplicons were purified from the gel with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced on the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the Bigdye Terminator V3.1 Cycle sequencing kit. The sequences obtained were analyzed in the Blastn programme found in the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov). The result of the analysis was decided according to the query coverage and e-values.

Antimicrobial sensitivity. The antimicrobial susceptibilities of gram-negative bacteria were assessed by the Kirby-Bauer disk diffusion method, as described by EUCAST [50]. The density of the bacterial suspension was adjusted by comparison with the 0.5 McFarland standard. This suspension was spread on Mueller-Hilton Agar medium (Oxoid, Basingstoke, Hampshire, UK). Seventeen commercial antibiotic discs: ampicillin (AMP; 10 µg), co-amoxiclav (AMC; 10 µg), ceftriaxone (CRO; 30 µg), cefoxitin (FOX; 30 µg), cefotaxime (CTX; 30 µg), trimethoprim/sulfamethoxazole (SXT; 25 µg), levofloxacin (LEV; 5 µg), ceftazidime (CAZ; 30 µg), cefepime (FEP; 30 µg), cefepime-aztreonam (ATM; 10 µg), piperacillin/ tazobactam (TZP; 10 µg), *c*iprofloxacin (CIP; 5 µg), gentamicin (CN; 10 µg), amikacin (AK; 10 µg), meropenem (MEM; 10 µg) and ertapenem (ETP; 10 µg), were placed on Mueller-Hinton Agar medium and incubated at 37 °C for 18–24 hours [51]. Antimicrobial susceptibility results were recorded by measuring the diameters of the zone of inhibition, and the data evaluated according to the standards of the European Committee for Anti-microbial Susceptibility Tests (EUCAST) [50].

Statistical analysis. The Mann-Whitney U test was used for all statistical analyses. Comparison between two groups (weight and size) and genders was performed by the Mann-Whitney U test. A p-value < 0.05 was considered statistically significant.

RESULTS

MALDI-TOF. The study included 54 male and 50 female sea snails, with their weights ranging between 11.24 g – 195.12 g, and size ranging between 41.16 mm – 92.14 mm. A significant relevance was observed between gender and body weight (p = 0.019), and gender and size (p = 0.010) of the sea snails. Overall, 24 different bacterial species were isolated from the samples by MALDI-TOF (Tab. 2). Bacterial growth was highly abundant in both the intestine (100%) and meat samples (94.2%). The most commonly isolated organisms were ***Aeoromonas salmonicida subsp. salmonicida*** at 33.7% (35/104), followed by ***Raoultella ornithinolytica*** (***R. ornithinolytica)*** at 9.6% (10/104) and ***Staphylococcus warneri (S. warneri)*** at 7.7% (8/104) (Tab. 2). Figure 2 represents the peptide mass fingerprint spectra of ***A. hydrophila*** compared with the reference spectra.

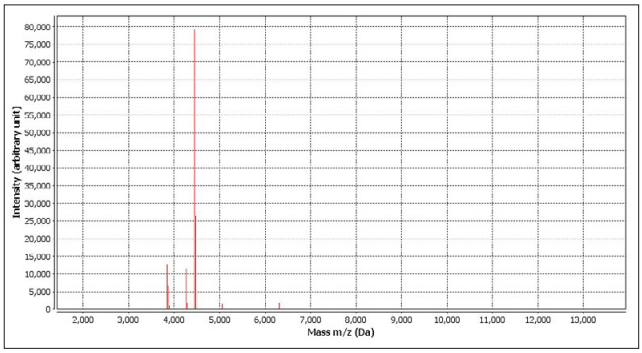

Figure 2. A peptide mass fingerprint spectrum of *A. hydrophila* by comparing with a database of the reference spectra

Table 2. Bacterial isolates obtained from the intestine and meat samples of the sea snails using MALDI-TOF MS techniques

BACTERIA	INTESTINE SAMPLES n (%)	MEAT SAMPLES n (%)
Acinetobacter lwoffii	5 (4.8)	5 (4.8)
Aeromonas salmonicida ssp salmonicida	35 (33.7)	35 (33.7)
Aeromonas enteropelogenes	2 (1.9)	2 (1.9)
Aeromonas hydrophila / punctata (caviae)	3 (2.9)	3 (2.9)
Aeromonas sobria	2 (1.9)	2 (1.9)
Bacillus altitudinis/pumilis	3 (2.9)	-
Bacillus amyloliquefaciens	2 (1.9)	-
Bacillus cereus group	2 (1.9)	2 (1.9)
Bacillus firmus	1 (1)	-
Enterococcus faecium	5 (4.8)	5 (4.8)
Klebsiella aerogenes	1 (1)	1 (1)
Klebsiella oxytoca	1 (1)	1 (1)
Lactococcus garviae	2 (1.9)	2 (1.9)
Paenibacillus provencensis	1 (1)	1 (1)
Pseudomonas mendocina	2 (1.9)	2 (1.9)
Raoultella ornithinolytica	10 (9.6)	10 (9.6)
Raoultella planticola	1 (1)	1 (1)
Shewanella putrefaciens	2 (1.9)	2 (1.9)
Staphylococcus aureus	2 (1.9)	2 (1.9)
Staphylococcus epidermidis	5 (4.8)	5 (4.8)
Staphylococcus hominis	7 (6.7)	7 (6.7)
Staphylococcus warneri	8 (7.7)	8 (7.7)
Streptococcus mitis/ Streptococcus oralis	1 (1)	1 (1)
Vibrio vulnificus	1 (1)	1 (1)
Total	**104 (100)**	**98 (94.2)**

Antimicrobial sensitivity. Kirby-Bauer disc diffusion assay showed that the isolates were antimicrobial-resistant. Susceptibility to AMP, AMC, CRO, FOX, CTX, SXT and ETP was observed in two *P. mendocina* isolates; only one *P. mendocina* isolate was resistant to LEV. Susceptibility to AMP, AMC, CRO, FOX, CTX, CAZ, FEP, ATM, TZP and ETP was observed in five *A. lwoffii* isolates (Tab. 3).

A total of 66 isolates were investigated for antimicrobial susceptibility testing. The highest percentage in *A. salmonicida* subsp. *salmonicida* were observed for AMP at 27/35 (77.1%), while the lowest resistance percentages were seen for the other antibiotics. *Aeromonas hydrophila/punctata (caviae)*, *A. sobria*, *K. aerogenes*, *K. oxytoca*, *R. planticola*, *S. putrefaciens*, and *V. vulnificus* were intrinsic resistant to ampicillin. *S. putrefaciens* had the highest resistance rate (100%) to CRO, CTX, CAZ, FEP, and ATM. *Vibrio vulnificus* were resistant to AMC, CRO, FOX, CTX, CAZ, FEP and SXT. *A. salmonicida* subsp. *salmonicida* showed very low levels of resistance to LEV and MEM at 1/35 (2.9%) and 1/35 (2.9%), respectively.

Multiplex PCR for *mcr*, carbapenemase and β lactamase resistance genes. A total of 66 isolates were used in a multiplex PCR assay to detect the presence of ***mcr*** and carbapenemase resistant genes, using the primers specific for ***mcr***, carbapenem and β-lactamase resistant genes. It was found that all of the subjected isolates were negative for bla_{TEM-1}, bla_{CTX-M}, bla_{SHV-1}, bla_{OXA-48}, bla_{NDM-1}, bla_{KPC}, bla_{IMP} and bla_{VIM} and ***mcr*** genes (Fig. 3).

Results by 16S rRNA sequence analysis. Based on the GenBank and the BLAST server in NCBI, *A. hydrophila/ punctata (caviae)* isolates were identified as *A. hydrophila*.

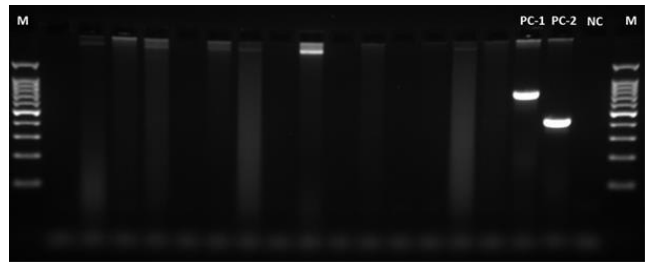

Figure 3. Agarose gel electrophoresis (1.5%) used for separation of the different multiplex products

Lanes labeled 'NC' correspond to blank controls, 'PC-1' correspond to KPC positive control (798 bp), 'PC-2' correspond to OXA-48 (438 bp) positive control. The isolated samples did not amplify for KPC, OXA-48, NDM, IMP, VIM resistance genes. Lanes labeled 'M' correspond to 100 bp molecular weight marker (Transgen Biotech, Beijing, China).

DISCUSSION

Intestinal microbiota of sea snails. The gut bacterial communities of snails are unclear [52] and a relatively small number of published studies are available which describe their intestinal microbiota. One such study focussed on culture-based methods and identified a small number of bacteria which included *Clostridium*, *Enterobacter*, *Enterococcus*, and *Lactococcus* [53]. Another study compared the gut bacterial diversity of herbivorous feeding (HV) and non-herbivorous feeding in the freshwater snail *Planorbella trivolvis* using culture-independent molecular analysis, and reported that gut microbiota in the herbivorous feeding

Table 3. Antibiotic resistance of bacterial isolates obtained from the intestine and meat samples of sea snails

BACTERİAL SPECİES	ANTİMİCROBİAL RESİSTANCE N (%)																
	AMP	AMC	CRO	FOX	CTX	CAZ	IMP	FEP	ATM	CN	TZP	SXT	CIP	LEV	MEM	AK	ETP
Acinetobacter lwoffii (5)	-	-	-	-	-	-	-	-	-	-	-	1 (20)	-	-	-	-	-
Aeromonas salmonicida ssp salmonicida (35)	27 (77.1)	10 (28.6)	10 (28.6)	7 (20)	10 (28.6)	10 (28.6)	-	9 (25.7)	9 (25.7)	8 (22.9)	-	9 (25.7)	3 (8.6)	1 (2.9)	1 (2.9)	2 (5.7)	7 (20)
Aeromonas enteropelogenes (2)	1 (50)	-	1 (50)	-	1 (50)	1 (50)	-	1 (50)	1 (50)	-	-	-	-	-	-	-	-
Aeromonas hydrophila/ punctata (caviae) (3)	3 (100)	-	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	-	-	1 (33.3)	1 (33.3)	-	-	-	-	-	1	1
Aeromonas sobria (2)	2 (100)	-	-	1 (50)	-	-	-	-	-	-	-	1 (50)	1 (50)	1 (50)	-	-	-
Klebsiella aerogenes (1)	1 (100)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Klebsiella oxytoca (1)	1 (100)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Paenibacillus provencensis (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas mendocina (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raoultella ornithinolytica (10)	7 (70)	2 (20)	3 (30)	2 (20)	3 (30)	3 (30)	-	2 (20)	2 (20)	1 (10)	-	3 (30)	-	-	-	-	-
Raoultella planticola (1)	1 (100)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Shewanella putrefaciens (2)	2 (100)	1 (50)	2 (100)	1 (50)	2 (100)	2 (100)	-	2 (100)	2 (100)	-	-	1 (50)	-	-	-	-	-
Vibrio vulnificus (1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	-	1 (100)	1 (100)	-	-	1 (100)	-	-	-	-	-

snails commonly consisted of *Proteobacteria* (52.97%) and *Bacteroidetes* (28.75%), whereas the gut microbiota in non-herbivorous feeding snails was commonly *Proteobacteria* (95.23%) [54]. The same researchers also indicated that *Cloacibacterium* (24.60%), OM6ON (6.12%), *Rhodobacter* (5.79%), and *Pseudomonas* (4.47%) were found to be abundant in herbivorous snails, and that in addition, *Aeromonas* (85.4%) was commonly detected in non-herbivorous feeding snails [54]. In the current study, the intestinal microbiota of snails were isolated and compared with the gender and the body weight of the snails. A significant relationship was found between gender and body weight ($p = 0.019$), and gender and size ($p = 0.010$) of the sea snails.

So far, 36 species autochthonous to aquatic environments have been identified within the genus *Aeromonas* and have also been obtained from animals, foods, and different human infections [55, 56]. Researchers have detected 96.5% of the strains from the clinical cases, among which only four species cause severe disease in humans – *A. caviae* (29.9%), *Aeromonas dhakensis* (26.3%), *A. veronii* (24.8%), and *A. hydrophila* (15.5%) [57, 58]. In the presented study, *A. salmonicida* were isolated from 33.7% of snail samples, which is in accordance with earlier studies in which *A. salmonicida* and *A. caviae* were isolated from human samples using molecular identification [59].

Occurance of *Aeromonas* spp in the mea. Many *Aeromonas* spp. were found in tilapia and salmonids and included *A. allosaccharophila*, *A. bestiarum*, *A. bivalvium*, *A. encheleia*, *A. hydrophila*, *A. salmonicida* and *A. veronii* using molecular identification [60, 61]. In the current study, *Aeromonas* spp. were isolated from snail intestines and meat which, formed the major proportions of the isolates. These findings are in alignment with previous studies where *Aeromonas* species were found in fish on a global scale. *Aeromonas* was often identified from shellfish, such as cockles, mussels, oysters, and shrimps by molecular identification methods [61–63].

Pathogenic bacteria in sea snails. Most published studies for the detection of pathogenic bacteria in sea snails in Turkey have focused on biochemical methods. In a study performed to detect bacteriological microflora, such as *E. coli*, fecal coliform, and *Salmonella* spp. in sea snail (*Rapana venosa*), samples obtained between June 2000 – November 2001 from the northern coast of the Marmara Sea, the highest bacteriological values were detected in August were *Salmonella* spp., which were found in two groups of samples [29].

Identification of bacteria using MALDI-TOF MS. In the present study, the MALDI-TOF MS method was utilized as an alternative to traditional methods in the identification of aquatic bacteria, and is one of the most encouraging technologies for microbiological analysis. This method has advantages, such as high accuracy and specificity, high adaptability to the bacteriology laboratory, ease of analysis of data, and providing fast results, which are in agreement with previous publications [64]. In the current study, the most commonly identified organism by the MALDI-TOF MS technique was *A. salmonicida* subsp. *salmonicida* at 33.7% (35/104), followed by *R. ornithinolytica* at 9.6% (10/104) and *S. warneri* at 7.7% (8/104). A study recently stated that the spread of the fish pathogen, *A. salmonicida* is a vector in microplastics found in the sea [65]. In the current study, the use of MALDI-TOF MS technique provided additional information for the identification of *S. hominis, A. lwoffii, E. faecium, S. epidermidis, A. hydrophila/punctata (caviae), B. altitudinis/pumilis, A. sobria, A. enteropelogenes, Bacillus cereus group, B. amyloliquefaciens, Shewanella putrefaciens (S. putrefaciens), S. aureus, L. garviae, P. mendocina, Bacillus firmus, K. aerogenes, K. oxytoca, P. provencensis, R. planticola*, *S. mitis/ S. oralis* and *V. vulnificus.* These bacteria still pose the risk of human infection, caused by the consumption of sea snails.

Identification of carbapenem resistance bacteria. Recently, carbapenem resistance has been documented to be spreading quickly and is a growing threat to public health [66]. Therefore, the development of new methods for accurate and rapid identification of resistance to carbapenem is urgently needed [67]. Carbapenemase resistance can be determined by several methods in clinical laboratories [68]. These methods comprise automated systems (VITEK 2) or the disc diffusion test, modified Hodge test, MICs, selective agar, E-tests or double-disc synergy testing, spectrometric, whole-genome sequencing, and molecular techniques [68]. Researchers reported that reference MIC levels are more sensitive in detecting carbapenem susceptibility using broth microdilution and agar dilution than disc diffusion, many automated systems, and E-test [68, 69]. EUCAST shows that ertapenem has high sensitivity and low specificity, while meropenem has good sensitivity-specificity in determining carbapenem resistance in Enterobacteriales [70]. Accurate detection by phenotypic methods of carbapenem-producing Enterobacteriales is difficult due to low carbapenems' MICs [66]. Therefore, molecular methods are the gold standard tests for the determination of carbapenemase-producing genes [71].

Use of molecular techniques in identification and their limitations. Molecular techniques, such as multiplex PCR and microarray, have been accepted as the reference standard for the quick detection of carbapenemase-producing genes. Molecular methods, like the multiplex PCR in the present study, helped in the rapid determination of certain carbapenemase resistance genes from the samples synergistic with other traditional methods, is in accordance with previous studies which have identified carbapenemases genes in *Enterobacteriales* [72]. The limitations of these techniques are the lack of sequence identity for genes [22, 73, 74]. Previously, various molecular methods have been used to detect bacteria which were weakly identified by phenotypic methods due to the occurrence and spread of important genes via plasmids, Class B metallo β-lactamase (IMP, VIM, and NDM) and OXA-48 [72, 75].

Antimicrobial resistance and public health importance. Food contaminated with sea snails may cause disease in humans through consumption and handling [75]. Ampicillin, sulfamethoxazole, erythromycin, streptomycin and neomycin are commonly used antimicrobials in Turkey in the treatment of bacterial diseases [68]. *Aeromonas hydrophila/punctata (caviae), A. sobria, K. aerogenes, K. oxytoca, R. planticola, S. putrefaciens* and *V. vulnificus* displayed the highest levels (100%) of resistance to

ampicillin. It is well known that marine bacteria (i.e., *Aeromonas* spp., *S. putrefaciens*, *Vibrio* spp, *K. aerogenes*, and *R. ornithinolytica)* exhibit intrinsic resistance to most beta-lactam antibiotics, including ampicillin. It has also been observed that *S. putrefaciens* exhibited the highest levels (100%) of resistance to cefepime, cefotaxime, ceftazidime, ceftriaxone and cefepime-aztreonam. *Vibrio vulnificus* showed the highest levels (100%) of resistant to co-amoxiclav, ceftriaxone, cefoxitin, cefotaxime, ceftazidime, cefepime and trimethoprim/sulfamethoxazole. *Aeromonas salmonicida* subsp. *salmonicida* (*A. salmonicida* subsp. s*almonicida)* showed ver*y* low levofloxacin and meropenem resistance levels at 1/35 (2.9%) and 1/35 (2.9%), respecively. A high resistance to sulfamethoxazole, ampicillin, and aztreonam was determined in all isolates in a study from Turkey [2]. The findings of the present study are concordance with earlier studies performed in aquaculture.

Research on integrated fish-duck farming in Guangdong Province, China indicated that *mcr-1* positive *E. coli* can be transmitted from animals to humans by the aquaculture supply chain [76]. Hence, the determination of *mcr*-1.1 in fish is a serious problem for public health, since resistance can be potentially disseminated to humans and animals [76, 77]. The international trade in fish might be a route for the transfer of *mcr* between countries [51].

CONCLUSIONS

The study documented that sea snails may act as a reservoir for the various antimicrobial resistant bacteria, which in turn helps in the dissemination of pathogenic and non-pathogenic bacteria in sea water. The aim of the study was to test for the presence of various antimicrobial resistant genes present in sea snails. No $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M}}$, $bla_{\text{SHV-1}}$, $bla_{\text{OXA-48}}$, bla_{NDM1}, bla_{KPC}, bla_{IMP}, bla_{VIM} and *mcr* genes in Gram-negative bacteria were found in the obtained snails. To the best of the knowledge of the authors, this study is the first to research $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M}}$, $bla_{\text{SHV-1,}}$ bla_{OXA}48, $bla_{\text{NDM-1}}$, bla_{KPC}, bla_{IMP}, bla_{VIM} and *mcr* genes in sea snails in Turkey and worldwide. The study implemented both molecular and biochemical methods to identify the absence of *mcr,* carbapenemase and beta-lactamase resistant genes in the intestine and meat of sea snails. There is a high risk for the spread of bacteria via aquatic environments, hence, serious precautions should be utilized to control the spread of pathogenic bacteria. Further advanced detection methods should also be used to detect antimicrobial resistant genes in sea snails. The results necessitate the requirement of experimental studies before the export of sea snails abroad, to evaluate the potential risks to human health by sea snails obtained from different geographical regions.

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