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Characterisation of the antimicrobial resistance profile of culturable Gram-negative bacterial isolates from green sea turtles (*Chelonia mydas)* in the Gulf of Thailand

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A R T I C L E I N F O

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A B S T R A C T

Green sea turtles are endangered globally; this is partly due to anthropogenic threats including environmental pollution. This study investigated antimicrobial resistance (AMR) in culturable Gram-negative bacteria from green sea turtles at a rehabilitation centre and wild-stranded green sea turtles in the Gulf of Thailand. Cloacal samples were collected from 126 captive and 13 wild green sea turtles, from which 47 Gram-negative bacterial isolates (24 captive and 23 wild) were identified. Among the identified isolates, *Citrobacter* spp. exhibited the highest prevalence (31.9 %), followed by *Alcaligenes faecalis* (8.5 %), *Proteus mirabilis* (8.5 %), and *Vibrio* spp. (6.4 %). Many isolates (76.6 %) were resistant to multiple antibiotics. The statistical analysis of AMR across 14 antibiotics revealed significant differences between captive and wild green sea turtles ($p = 0.0329$). A significantly higher incidence of resistance to cefoxitin ($p = 0.0184$), ampicillin ($p = 0.0027$), and amoxicillin-clavulanic acid ($p = 0.0255$) was observed in captive turtles compared to wild turtles. In contrast, wild turtles exhibited significantly higher resistance to potentiated sulfonamides ($p = 0.0388$) and tetracyclines ($p = 0.0002$). These findings indicate that antibiotics that are commonly used in human and veterinary medicine, aquaculture and agriculture are exerting selection pressure on gut bacteria in green sea turtles in Thailand, leading to the development of AMR. While the use of antibiotics to manage infections in turtle rehabilitation facilities is common, selection for AMR in wild green sea turtles may result from anthropogenic activities leading to environmental contamination with antibiotics and other biocides. Strategies to mitigate this problem are urgently needed.

1. Introduction

Antibiotics are widely used in medicine, agriculture, and livestock industries to prevent and treat bacterial infections (Qiao et al., 2018). This leads to a persistent release of antibiotics into the environment and leads to the proliferation of antibiotic resistant bacteria (ARB) expressing antibiotic resistance genes (ARGs) (Zhang et al., 2009). Antimicrobial resistance (AMR) significantly reduces the therapeutic efficacy of antibiotics and also alters ecosystem microbial communities. This poses a serious threat to global public health and can markedly impact the environmental ecology (Gomes, 2024). This is facilitated by the horizontal transfer of ARGs among bacteria, and the ecological recycling of ARB and ARGs at the human-animal-environment interface. For public health, the increase in AMR incidences undermines the effectiveness of current antimicrobial therapies, which complicates disease management and increases the burden on healthcare systems (Anthony et al., 2020). In 2019, the World Health Organisation (WHO) listed AMR among the top ten threats to global health. This challenge, however, extends beyond a direct impact on humans, reaching into marine environments and intricately affecting ecosystem well-being and wildlife species (Sanjeev et al., 2023). In green sea turtles, the presence of ARB in the gut microbiome poses a threat to normal physiological function and

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health. These bacteria can cause infections that are difficult to treat with conventional antibiotics, potentially leading to increased mortality rates among turtles (Garcês and Pires, 2023). For instance, Delli Paoli Carini et al. (2017) demonstrated that ARB present in hospitalised green sea turtles and their contaminated holding tank water were associated with increased treatment challenges (Delli Paoli Carini et al., 2017).

The Gulf of Thailand is a vital habitat for green sea turtles, providing essential feeding and nesting grounds (Chomchat et al., 2024). However, the population of green sea turtles in this region has experienced a dramatic decline, with nesting rates falling to less than 50 % of levels documented in 1995 (Pradip Na Thalang et al., 2023). The actual causes of the decline in population and nesting for green sea turtles are still unknown (Prampramote et al., 2021). Human activities, including agriculture, aquaculture, urban development, and extensive industrial operations such as petrochemical plants, oil refineries, and coal-fired power stations, have significantly altered the marine ecosystem of the Gulf of Thailand. These activities cause significant environmental contamination by introducing heavy metals, volatile organic compounds, and other chemical byproducts into the ecosystem (Wattayakorn, 2006). This pollution directly degrades marine habitats, but it is also known to create conditions that favour the emergence and proliferation of ARB (Anand et al., 2021). For instance, exposing environmental bacteria to heavy metals, antibiotics and other biocides is known to activate SOS signalling pathways that lead to the co-selection of ARB by increasing the expression of ARGs (Engin et al., 2023). In addition, green sea turtles are also susceptible and are likely to manifest with cumulative effects of environmental contaminants due to their long lifespans and migratory behaviour [14]. In the Gulf of Thailand, green sea turtles are occasionally found stranded on the seashore, or in surrounding shallow waters, showing signs of illness or abnormal behaviour (Prampramote et al., 2022). Stranded and captured green sea turtles with health complications are kept in rehabilitation centres (Escobedo-Bonilla et al., 2022), where they are frequently treated with broad-spectrum antibiotics to manage microbial infections (Delli Paoli Carini et al., 2017). Both Gram-positive and Gram-negative bacteria cause infections in sea turtles, but majority of infections are caused by Gram-negative bacteria, which are normally present in the environment or as part of the turtles' bacterial flora. The sick and stranded green sea turtles can carry prolific pathogenic bacteria and pose a health risk to other living organisms sharing the same environment, due to their pathogenic potential and the possible dissemination of antibiotic resistance in marine ecosystems within the world's oceans (Pace et al., 2019).

The extent of AMR in culturable Gram-negative bacteria in wild and captive green sea turtles has been categorised in different geographical locations (Drane et al., 2021b). Many studies have utilised culture and sensitivity testing for phenotypic assessment of AMR and it is cost-effective (Webber et al., 2022). This approach provides actionable data for guiding treatment decisions, while genomic and transcriptomic analyses detect ARGs and their expression patterns, and provide mechanistic insights, but do not confirm functional, phenotypic expression of AMR (Sukhum et al., 2019). To date, only one study has examined AMR associated with sea turtles in the Gulf of Thailand, and the focus was on AMR in rearing water tanks at the Sea Turtle Conservation Center of Thailand, with limited attention to gut bacteria in turtles (Chuen-Im et al., 2021). The current study aimed to investigate the incidence of AMR in culturable Gram-negative bacteria in cloacal swab samples from captive and wild green sea turtles in the Gulf of Thailand.

2. Material and methods

2.1. Ethics statement

The collection of samples from wild and captive green sea turtles was carried out with the approval of James Cook University Animal Ethics Committee (Permit no. A2931), Mahidol University Animal Ethics Committee (Permit no. MUVS-2023-10-66), and the Department of Fisheries, Bangkok, Thailand (Permit no. KS0510.54). Green sea turtles were restrained to collect cloacal swabs without the administration of anaesthesia. The animals were carefully handled to minimise distress and ensure their well-being under the supervision of experienced Thai aquatic veterinarians.

2.2. Study site

The samples were collected at the Sea Turtle Sanctuary (Lat.:12°36′54.9″N; Long.:101°41′23.3″E). This facility is part of the Royal Sea Turtle Conservation Project under the initiative of Her Majesty Queen Sirikit. It is located on Ko Man Nai Island in the Rayong Gulf of Thailand. This sanctuary is crucial in conserving endangered sea turtle species by providing a safe environment for nesting, hatching, and rehabilitation of injured sea turtles.

2.3. Animal categorisation

This study involved 139 green sea turtles which were categorised into captive (n = 126) and wild groups (n = 13). The captive turtles composed of juveniles (mean curved carapace length [CCL] 29.3 ± 2.6 cm, weight 3.14 ± 0.73 kg), which were reared from hatchlings obtained from Kram Island, and adults (mean CCL 63.4 ± 13.5 cm, weight 48.8 ± 31.6 kg), rescued from entanglement in fishing gear in the coastal regions of Chonburi (13.1701° N, 100.5611° E), Rayong (12.6926° N, 101.1777° E), Chanthaburi (12.6039° N, 102.0915° E), and Trat (12.4102° N, 102.4630° E). The wild group consisted of stranded turtles, which were often found floating or entangled in fishing gear and brought to the sanctuary from the same coastal areas. Upon arrival, the turtles underwent medical assessments and treatments. Due to logistical constraints under field conditions prior to sampling, body weight and demographic data were not recorded for some turtles in the wild group. The CCL was recorded for only 4 out of 13 stranded animals and these had an average length of 52.5 ± 7.4 cm. Morphometric data, including animal body weight and CCL were recorded according to procedures outlined by the Queensland Department of Environment and Heritage Protection Brisbane, Australia (https://environment.desi.qld.gov.au/). Available details on animal grouping and morphometric data are provided in Supplementary Table 1.

2.4. Samples collection

Samples from green sea turtles were collected in February 2024 and May 2024 (Supplementary Table 1). Deep cloacal swabs were collected from individual green sea turtles in both groups (captive and wild) and then randomly and evenly allocated to 5 pools for captive turtles and 3 pools for wild turtles. Each pooled sample was created in 50 mL of Tryptic Soy Broth (TSB) in Falcon tubes with slight agitation. To minimise the risk of exterior contamination, the cloacal area was flushed with 70 % ethanol before sample collection. A sterile polyester swab was then carefully inserted approximately 10 cm into the cloaca and gently rolled to ensure adequate sampling of the cloacal contents (Ahasan et al., 2017). The samples were kept in an icebox to maintain a temperature of approximately 4 °C and then transported to the laboratory at Kasetsart University (Bangkok, Thailand) within 13 h.

2.5. Bacterial culture and initial identification

The broth from each pooled sample for each group was serially diluted using PBS in 96-well plates according to a previously described procedure (Pariseau et al., 2024). The diluted samples (10^{-1} to 10^{-8}) were plated on MacConkey agar (MCK) plates (Merck Microbiological Media, Germany), containing 1.5 % NaCl to isolate culturable Gram-negative bacteria. The samples were also plated on MCK plates containing 3 % NaCl to maximise the isolation of culturable

Gram-negative bacteria capable of growing under higher salt conditions. The plates were incubated for 24–72h at 25 °C, and then plates with well-isolated colonies were selected. One colony for each observed morphology was picked up and subcultured in triplicate to ensure purity as outlined previously (Blasi et al., 2020). The isolates were then subjected to identification and antibiotic sensitivity testing.

2.6. MALDI-TOF mass spectrometric identification of bacteria

The purified isolates were cultured on tryptic soy agar (TSA), and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) was conducted using the VITEK® MS platform (Vitek MS, bioMérieux), to identify the isolates. Sample preparation followed the standard protocol described previously (Rodrigues et al., 2017). Each microbial isolate was analysed in duplicate to ensure reproducibility. The mass spectra generated from each run were automatically compared against a comprehensive reference database integrated within the MALDI VITEK® MS V3.1 system software (Cavalieri et al., 2019). For bacterial isolates that could not be identified using MALDI-TOF MS, 16S rRNA gene sequencing was performed for identification.

2.7. Molecular identification of bacterial isolates via 16S rRNA gene sequencing

Colonial purity of unidentified bacterial isolates was ensured by serial culturing of single representative colonies at least 3 times. Genomic DNA of each bacterial isolate was extracted for polymerase chain reaction (PCR) assays using the boiling method as described previously (Dunbar, 2023). The nucleic acid sequence of the 16S rRNA gene was amplified with a set of universal primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′ TACGGYTACCTTGTTACGACTT 3′). A Bio-Rad Thermal Cycler (Bio-Rad Laboratories Inc., USA) was used for PCR amplification. The standard PCR protocol utilised was as follows: initial denaturation at 95 °C for 5 min, followed by 31 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1.4 min. A final extension at 72 °C was performed for 10 min, and the samples were maintained at 4 °C until further processing. The presence of the DNA fragment was confirmed on 1 % agarose gel electrophoresis and visualised using GelRed under UV illumination. Following confirmation, the PCR products were purified and sequenced by Sanger sequencing using the Big Dye Terminator v3.1 cycle sequencing kit on the 3730XL automated DNA sequencing system. (Macrogen Inc., Seoul, South Korea). The Nucleotide sequences were analysed and aligned in Geneious (Biomatters Ltd.), followed by NCBI nucleotide BLAST identification (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.8. Antibiotic susceptibility test (AST)

Antibiotic susceptibility testing (AST) was performed on isolates to determine the phenotypic AMR profiles using the Kirby Bauer disc diffusion technique on Mueller–Hinton agar. A total of 14 distinct antimicrobial discs from different classes were used in the susceptibility testing. Briefly, a Mueller-Hinton agar plate was completely covered with an isolate onto which different antimicrobial discs were added. The tested antimicrobial classes included penicillins (amoxicillin-clavulanic acid [AMC, 30 μg], ampicillin [AMP, 10 μg]), cephalosporins (cefepime [FEP, 30 μg], ceftazidime [CAZ, 30 μg], cefoxitin [FOX, 30 μg]), aminoglycosides (gentamicin [CN, 10 μg]), carbapenems (imipenem [IMP, 10 μg]), rifamycins (rifampicin [RD, 5 μg]), folate synthesis inhibitors (trimethoprim [W, 5 μg], trimethoprim/sulfamethoxazole [TS, 25 μg]), tetracyclines (tetracycline [TE, 30 μg]), monobactams (aztreonam [ATM]), fluoroquinolones (ciprofloxacin [CIP, 5 μg]), and chloramphenicol (C, 30 μg). The zone of inhibition was measured and compared with quality control standards to determine a resistance/susceptible phenotype in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST Version 14.0, 2024; http://www.eucast.org) and Clinical and Laboratory Standards Institute (CLSI, 2020; https://clsi.org/) guidelines. As no standardised susceptibility breakpoints exist for *Shewanella* spp. in international guidelines, the susceptibility for isolates in this genus was evaluated using interpretative criteria established for *Pseudomonas aeruginosa*, which is a closely related strain (Song et al., 2021). Quality control was ensured using *Escherichia coli* (ATCC® 25922™) and *Staphylococcus aureus* (ATCC® 25923™) as reference strains.

2.9. Extended spectrum beta-lactamase (ESBL) detection

The isolates with inhibition zone size ≤22 mm with CAZ (30 μg) were considered ESBL-producers and these were subjected to further phenotypic examination as described previously. Briefly, a double-disc synergy test (DDST) was used to confirm ESBL production. A suspension of the test isolate was compared with McFarland standard (0.5 %) and plated on Mueller Hinton agar. CAZ (30 μg) discs were applied on agar 1.5 cm away from the centre of AMC (20 μg/10 μg) disc and incubated at 25 °C for 24–48 h. Any increase in the zone of inhibition towards the AMC disc (20/10 μg) was considered a positive result for ESBL enzyme production (Mohamed et al., 2020).

2.10. Screening for AmpC β lactamase producing strains

Bacterial strains were screened for expression of AmpC beta-lactamases by using the disc diffusion method that utilises FOX (30 μg) discs. Isolates with an inhibitory zone diameter of ≤18 mm were suspected to be AmpC β-lactamase producers. To confirm the AmpC β-lactamase production, a disc approximation assay (D Test) was also performed. The test isolate suspension was adjusted to 0.5 % McFarland standard and then plated on Mueller-Hinton agar. A disc containing CAZ (30 μg) was then placed adjacent to discs containing AmpC inducer antibiotics such as IMP (10 μg), FOX (30 μg), and AMC (30 μg), followed by incubation at 25 °C for 24–48 h. The appearance of a blunted or D shaped inhibitory zone around the CAZ (30 μg) disc towards the side of one of the inducers was considered a positive result for inducible AmpC β-lactamase production (Mohamed et al., 2020).

2.11. Statistical analysis

Descriptive and non-descriptive approaches were used to analyse and present results. Fisher's exact test was conducted using GraphPad Prism to compare antibiotic resistance patterns between wild and captive green sea turtles. Statistical significance was defined when p-value ≤0.05.

3. Results

3.1. Bacterial isolates

A total of 53 culturable Gram-negative bacterial isolates were obtained from both captive and wild green sea turtle populations. Out of 24 isloates in the captive group, *Citrobacter* spp. were the most prevalent, followed by *Vibrio anguillarum*, *Pseudomonas putida*, and *Shewanella putrefaciens*. Among the 23 isolates found in the wild group, *Alcaligenes faecalis* and *Proteus mirabilis* were the dominant species, followed by *Aeromonas* spp. and *Citrobacter* spp. Notably, five bacterial isolates from wild green sea turtles remained unidentified and one additional isolate was classified as *Xanthomonas* spp., a flora associated pathogen. The species and genus of most isolates was determined using MALDI-TOF-MS; however, *Pseudomonas plecoglossicida, Paenalcaligenes suwonensis, Vibrio anguillarum,* and *Hafnia paralvei* could only be identified via 16S rRNA gene sequencing. All culturable Gram-negative bacteria that were isolated in this study are presented in Fig. 1.

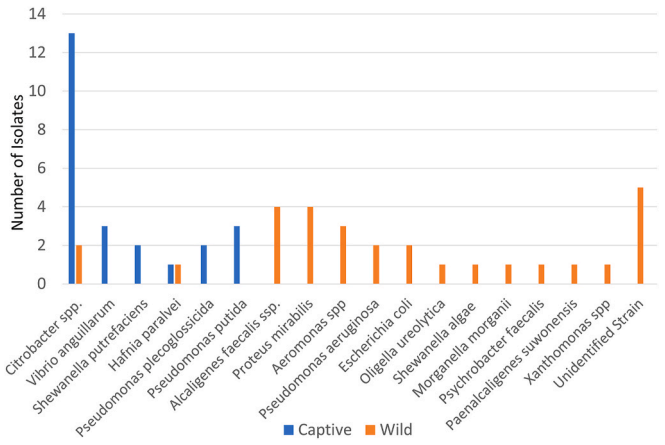

Fig. 1. The figure illustrates the composition of culturable Gram-negative bacterial isolates from cloacal swabs of captive and wild green sea turtles in in the Gulf of Thailand. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Antibiotic resistance patterns in all identified gram negative isolates

The resistance profiles of bacterial isolates from both wild and captive green sea turtles were assessed across various antibiotic classes. The isolates exhibited high resistance to penicillins, with 78.8 % of isolates showing resistance against AMP and 69.7 % against AMC. Varied incidences of resistance against cephalosporins were observed and FOX had the highest incidence (84.8 %). Moderate incidences of resistance were seen against carbapenems and aminoglycosides, while resistance to folate pathway inhibitors and phenicols was notably high. Resistance to other classes, such as fluoroquinolones and tetracyclines, was relatively low. Detailed antimicrobial resistance profiles are shown in Fig. 2 (a) and 2 (b).

3.2.1. Antimicrobial resistance profiles in captive and wild green sea turtles

AMR profiles of bacterial isolates from green sea turtles (*Chelonia mydas*) revealed significant differences between captive and wild sea turtles. Isolates from wild sea turtles exhibited higher resistance to FOX, with 100 % (17/17) resistance compared to 68.8 % (11/16) in captive sea turtles ($p = 0.0184$). In contrast, AMP resistance was significantly higher in captive sea turtles at 100 % (17/17) versus 56.3 % (9/16) in wild turtles ($p = 0.0027$). Resistance to AMC was also elevated in captive sea turtles at 88.2 % (15/17) compared to 50 % (8/16) in wild sea turtles ($p = 0.0255$). However, resistance to both W and C was significantly higher in wild sea turtles at 93.8 % (15/16) and 81.3 % (13/16), respectively, compared to 35.3 % (6/17) and 29.4 % (5/17) in captive turtles ($p = 0.0008$). Resistance to IMP was comparable across both groups, with rates of 50 % (12/24) in captive sea turtles and 47.8 % (11/23) in wild sea turtles ($p > 0.9999$).

Notably, resistance against TE was minimal in captive sea turtles 5.9 % (1/17) but significantly higher in wild sea turtles 68.8 % (11/16) ($p = 0.0002$). The incidence of resistance against CIP was 12.5 % (3/24) in captive sea turtles and 47.8 % (11/23) for wild sea turtles ($p = 0.0114$). Incidences for resistance against FOX ($p = 0.0184$), AMP ($p = 0.0027$), and AMC ($p = 0.0255$) were significantly higher in captive sea turtles, while wild sea turtles exhibited higher incidences of resistance against W ($p = 0.0388$), TS and TE. RD had the lowest incidence of resistance in both groups (captive: 5.9 %; wild: 12.5 %). All these data are summarised in Fig. 3. Overall, significant differences in antimicrobial resistance across 14 antibiotics were observed between captive and wild sea turtles ($p = 0.0329$).

3.2.2. Multidrug resistance patterns in bacterial isolates from green sea turtles

Among 47 Gram-negative bacterial isolates (24 from captive turtles and 23 from wild turtles), varying levels of multidrug resistance were observed. Approximately 15 % (7/47) of the isolates were resistant to a single antibiotic but 6.4 % (3/47) of the isolates were resistant to two antibiotics, and 76.6 % (36/47) exhibited resistance to multiple (≥ 3) antibiotics and these were regarded as multidrug resistant isolates (MDR). Multidrug resistance was observed in all *Citrobacter* spp. (13/13) and *Vibrio* spp. (3/3) isolated from captive sea turtles, as well as in most *Pseudomonas putida* (2/3). One *Hafnia paralvei* isolate exhibited pan-drug resistance. In wild sea turtles, multidrug resistance was observed in all *Alcaligenes faecalis* and *Proteus mirabilis* isolates (4/4), as well as *Escherichia coli* (2/2), *Citrobacter* spp. (2/2), and *Pseudomonas aeruginosa* (1/2). A detailed outline of all MDR isolates is provided in Supplementary Table 2.

3.2.3. Expression of ESBL and AmpC in tested bacterial isolates

The AMR profiling of bacterial isolates from captive and wild green sea turtles revealed the presence of AmpC β-lactamase and ESBL producers in some of the isolates. Among the 28 tested isolates, 15 (53.6 %) were identified as AmpC β-lactamase producers and this included 12 isolates (42.9 %) from captive sea turtles and 3 isolates (10.7 %) from wild sea turtles. ESBL activity was detected in 3 of the 9 isolates tested (33.3 %) and this included 2 isolates (22.2 %) from captive sea turtles and 1 isolate (11.1 %) from wild sea turtles as detailed in Supplementary Table 3.

4. Discussion

The increasing prevalence of AMR in marine ecosystems poses a significant threat to both wildlife health and conservation efforts for endangered species (Singh et al., 2022). Current research on green sea turtles in Thailand focuses on husbandry in captivity, genetics, blood biochemical profiles, and the impact of macroplastic debris. Studies on AMR in bacteria isolated from sea turtles are limited (Chuen-Im et al., 2021). Previous research revealed that the Sea Turtle Conservation Center of Thailand (STCCT) faced a major problem of bacterial infections in rearing turtles. The identification of bacterial isolates from lesions on tissues or organs in carcases of juvenile turtles revealed that most etiologic agents belonged to the families *Vibrionaceae*, *Staphylococcaceae*, and *Enterobacteriaceae*, where the corresponding predominant genera were *Vibrio*, *Staphylococcus*, and *Citrobacter*. These bacteria were also commonly found in seawater from the juvenile green sea turtle rearing tanks but they have the potential to serve as primary pathogens, or opportunistic bacteria causing diseases in sea turtles (Chuen-Im et al., 2021). In terms of pathogenicity, Gram-negative bacteria are generally more virulent than Gram-positive bacteria, and AMR is frequently reported in Gram-negative bacteria isolated from both wild and captive green sea turtles (Tsai et al., 2021). The distribution of Gram negative bacteria isolated in the present study was relatively similar to existing evidence, indicating that different species of Gammaproteobacteria and Betaproteobacteria represent the dominant bacterial classes in cloacal samples of green sea turtles (Ahasan et al., 2017). The high prevalence of *Citrobacter* species in captive green sea turtles may be attributed to their ubiquitous presence in diverse environments, such as soil, water, and the intestines of animals (Chen YingSheng et al., 2002). *Citrobacter freundii* and *C. braakii* have been linked to diseases like arthritis, coelomitis, and ulcerative stomatitis in green sea turtles (Ebani, 2023). *Pseudomonas putida* and *Pseudomonas plecoglossicida* were isolated from captive sea turtles, while *Pseudomonas aeruginosa* which is a well-documented opportunistic pathogen was found in wild sea turtles. *Pseudomonas* species, including *P. aeruginosa* and *P. putrefaciens*, act as opportunistic pathogens, often following traumatic injuries, and have been associated with diseases such as fibropapillomatosis (Ebani, 2023). *Vibrio anguillarum* was associated with wound infections and mortalities in Olive

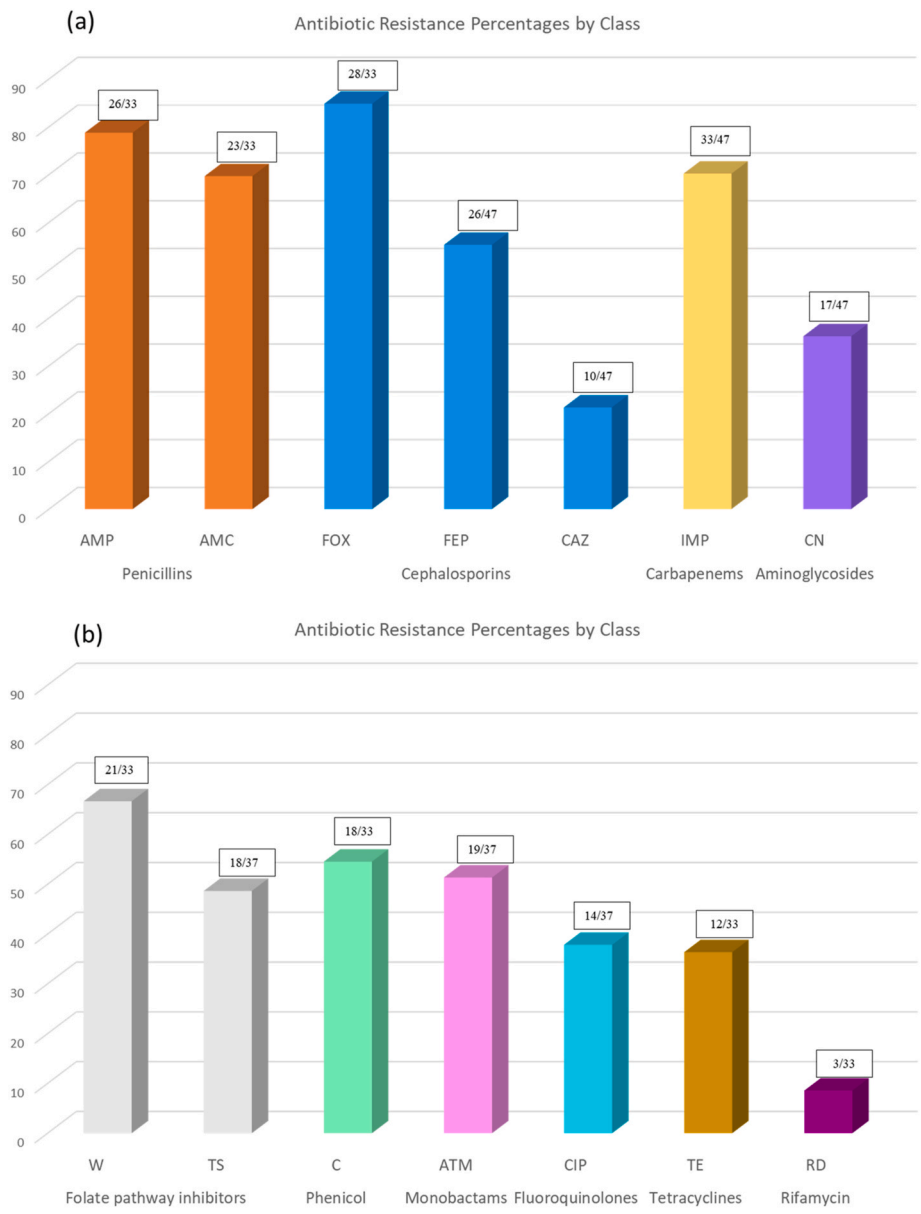

Fig. 2. Antibiotic resistance patterns in bacterial isolates from wild and captive green sea turtles, showing resistance profiles across different antibiotics. Panel (a) shows incidences of resistance against ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefoxitin (FOX), cefepime (FEP), ceftazidime (CAZ), imipenem (IMP), and gentamicin (CN). Panel (b) shows incidences of resistance against trimethorprim (W), trimethoprim/sulfamethoxazole (TS) chloramphenicol (C), aztreonam (ATM), ciprofloxacin (CIP), tetracycline (TE), and rifamycin (RD). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Ridley sea turtles (Ebani, 2023), thereby indicating a potential role in infections among green sea turtles. *Alcaligenes faecalis was* previously isolated from turtle nesting sands (Candan and Candan, 2020), while *Proteus mirabilis* naturally occurs in the gastrointestinal tract microbiota of sea turtles and other marine animals and has also been associated with ill health in turtles (Short et al., 2023). *Hafnia* species including (*alvei* and *paralvei*) are rarely reported in sea turtles. However, the potential for *Hafnia* spp. to cause severe infections in immunocompromised human hosts has been reported, and the pathogenicity of these strains remains poorly understood (Yin et al., 2019). Other emerging pathogens have been isolated from turtles in other studies and these include *Paenalcaligenes suwonensis*, linked to acute gastroenteritis (Olowo-Okere et al., 2020), and *Oligella ureolytica*, implicated in fatal human infections (Serandour et al., 2023). Collectively, these findings highlight the potential zoonotic risks posed by sea turtle microbiota, as these pathogens may cross species barriers.

In addition to carrying potentially opportunistic pathogenic bacteria, an additional challenge to turtle health worldwide, is the exposure of gut bacteria to antibiotics and other biocides that exert selection pressure leading to the development of AMR. As a result, marine pollution has been identified as a priority research area for global sea turtle conservation, and the presence of ARB in marine turtles may be indicative of coastal contamination (Tsai et al., 2021). The Gulf of Thailand is an example of an ecosystem that may be impacted by different epidemiological drivers of AMR. The coastal areas around this Gulf are influenced by the discharge of pollutants from several rivers, including the Chao Phraya, Tha Chin, Mae Klong, Bang Pakong, Prachin Buri, and Rayong, which carry high levels of pollutants from agricultural, industrial, and domestic activities (Wattayakorn, 2006). In addition, the Map Ta Phut industrial hub with its industrial estates, ports, and factories, is a major source of pollutants, including heavy metals, nutrients, and volatile organic compounds, and all these anthropogenic activities have the

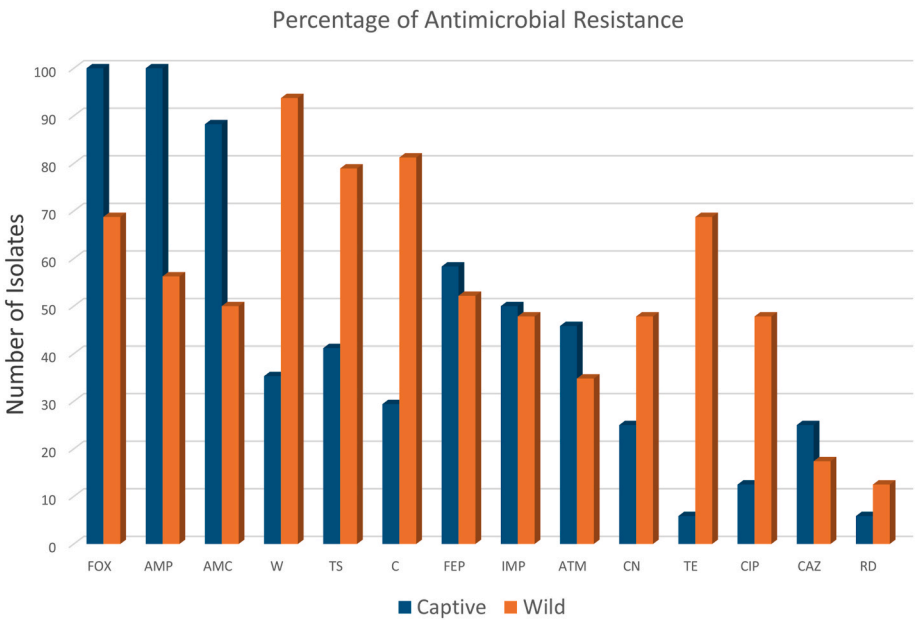

Fig. 3. Illustration of antimicrobial resistance percentages in captive and wild green sea turtles. Abbreviations are defined as: FOX = cefoxitin; AMP = ampicillin; AMC = amoxicillin-clavulanic acid; FEP = cefepime; CAZ = ceftazidime; IMP = imipenem; CN = gentamicin; W = trimethoprim; TS = trimethoprim/sulfamethoxazole; C = chloramphenicol; ATM = aztreonam; CIP = ciprofloxacin; TE = tetracycline; RD = rifamycin. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

potential to significantly impact the Gulf's marine ecosystems (Burnett et al., 2019). This continuous exposure to pollutants is known to exert selective pressure on marine bacteria, fostering the development of antibiotic resistance (SR and Sumithra, 2023). Additionally, heavy metals and other biocides in these effluents can co-select for AMR genes, further exacerbating the issue (Engin et al., 2023). Nonetheless, to the best of our knowledge, no previous study has investigated AMR of culturable Gram-negative bacteria isolated from green sea turtles in the turtle sanctuary studied herein, and its coastal surroundings in Gulf of Thailand.

Identified, culturable Gram-negative isolates from cloacal swabs of green sea turtles in the Gulf of Thailand exhibited marked resistance against all tested antimicrobial compounds and, majority (77 %) were MDR isolates. The profiles of MDR isolates in the current study are similar to those reported for *Citrobacter* spp., *Vibrio* spp., *Aeromonas* spp., *Morganella morganii*, *Proteus mirabilis*, and *Escherichia coli* in previous studies (Ahasan et al., 2017; Tsai et al., 2021). However, to the best of our knowledge, this seems to be the first documented report of MDR in *Alcaligenes faecalis*, *Hafnia paralvei*, *Paralcaligenes suwonensis*, and *Oligella ureolytica* isolated from cloacal swabs of green sea turtles. In addition, the detection of AmpC and evidence for ESBL production in *Citrobacter* spp. and *Pseudomonas* spp. raises serious concerns over the induction and dissemination of ARGs that can code for resistance across multiple antibiotics. This indicates that wild and captive green sea turtles in the Gulf of Thailand may be critical reservoirs of AMR in marine ecosystems. More specifically, the occurrence MDR isolates in captive and wild green sea turtles, and pan-drug resistant isolates in captive juvenile turtles presents a significant ecological concern. We show that captive sea turtles without any known or documented prior exposure to antibiotics exhibited complete resistance to FOX and AMC, with substantial resistance to FEP within the beta-lactam class. Our findings on AMR are consistent with a worldwide review that identified β-lactams as the most affected antimicrobial class in green sea turtles (Drane et al., 2021a). Similarly, a study from Taiwan reported high resistance in cloacal isolates to penicillins (amoxicillin, 78 %; amoxicillin–clavulanic acid, 39 %), macrolides (spiramycin, 68 %), and cephalosporins (cephalexin, 67 %; cefoperazone, 43 %) (Tsai et al., 2021). Comparable AMR patterns have also been documented in other sea turtle species including juvenile loggerhead (*Caretta caretta*), green (*Chelonia mydas*), black (*Chelonia agassizii*), and olive ridley turtles (*Lepidochelys olivacea*) (Chuen-Im et al., 2021). Detection of these high incidences of AMR in sea turtles with no evidence for direct exposure to antibiotic treatment suggests environmental contamination as a likely factor. Specifically, the water in captivity tanks, sourced from the Gulf of Thailand, may be polluted with antibiotic residues or ARB that may facilitate the acquisition of resistance genes by the resident microbiota via horizontal gene transfer mechanisms. This hypothesis aligns with findings from the STCCT, where rearing seawater was identified as a reservoir for ARB, posing health risks to captive sea turtles (Chuen-Im et al., 2021). To our knowledge, the wild green sea turtles included in this study did not receive any antibiotics prior to sampling. It was anecdotally reported by the attending aquatic veterinarian however, that stranded animals are commonly treated with enrofloxacin and trimethoprim/sulfamethoxazole when antimicrobial therapy is required. The observed AMR profiles indicated that in stranded, wild green sea turtles, there was a significantly higher incidence of resistance against a broader class of antimicrobial compounds including trimethoprim, chloramphenicol, trimethoprim/Sulfamethoxazole and tetracycline. These differences in resistance patterns between captive and wild green sea turtles may be attributed to variations in exposure to antibiotics and other biocides. Captive sea turtles are more likely to receive direct antibiotic treatment in rehabilitation facilities, whereas wild sea turtles may acquire resistant bacteria from polluted coastal waters during their long-distance migrations.

The implications of resistance to reserved and critically important antibiotics, such as gentamicin, chloramphenicol, cefepime, imipenem, aztreonam and rifampicin extends beyond sea turtle rehabilitation or conservation, as this poses a serious public health threat. These drugs are among the essential last resort treatments for many life threatening infections in human and veterinary medicine. For instance, cefepime and imipenem tend to be among the few effective options against infections caused by problematic MDR, ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.) pathogens. Thus, the observed resistance against reserved antibiotics in green sea turtles in the Gulf of Thailand may contribute to the proliferation of resistant bacterial strains

in the environment. This would markedly complicate any therapeutic interventions if humans and other animals are exposed to these MDR isolates. Collectively, our observations highlight the complex pathways through which anthropogenic activities drive microbial shifts, raising concerns over ecological stability and AMR transmission in marine ecosystems. However, this study carries a few limitations. This analysis was restricted to culturable Gram-negative isolates under specific laboratory growth and transport conditions described herein. Thus, the isolates may provide an incomplete representation of the microbial community in green sea turtles. Many microorganisms, particularly those in the gut microbiome, are not culturable and this potentially excluded critical contributors to the observed AMR profiles. Moreover, the molecular mechanisms underlying most of the observed AMR patterns remain undefined and as such, a deeper elucidation of ARG transfer and expression dynamics was not done. To alleviate logistical as well as project timeline constraints, cloacal swab samples from green sea turtles were pooled as opposed to culturing individual samples, which may have possibly limited the number of bacterial isolates that were extracted and identified. Additionally, metadata including weight and curved carapace length were unavailable for some wild green sea turtles due to challenging field conditions and this complicates the complete stratification of our data according to demographic details. Future studies should prioritise more comprehensive demographic and captivity data collection. Cloacal samples may represent the fecal microbiome but not the entire gastrointestinal mucosal bacteria of sea turtles; these aspects were beyond the scope of the current study. It is possible as well as rational therefore, to design future studies aimed at addressing these limitations.

5. Conclusion

In conclusion, this study investigated the antibiotic resistance profile of culturable Gram-negative bacteria isolated in cloacal swab samples from captive and wild green sea turtles in the Gulf of Thailand. This study revealed resistance to beta-lactam drugs was significantly higher in captive sea turtles while wild sea turtles demonstrated high resistance to folate pathway inhibitors and tetracyclines. The lowest resistance was observed to rifampicin in both groups. Accordingly, antibiotic-resistant bacteria should be considered as a major health concern for sea turtles, and monitoring AMR should be implemented for marine environments and in microbiological analysis of water quality for sea turtle rehabilitation facilities. The identification of MDR Gram-negative bacteria in both captive and wild green sea turtles highlights the concerning extent of antimicrobial contamination in coastal waters around the Gulf of Thailand. Results from this study provide baseline information on AMR in green sea turtles and the potential contribution of anthropogenic activities in the development of AMR in the Gulf of Thailand. We identify critical gaps in knowledge and research that is needed to fully evaluate the level of contamination and impact of antibiotics, biocides and other pollutants in the Gulf of Thailand, but also highlight that strategies to mitigate these problems are urgently needed.

CRediT authorship contribution statement

Dawood Ghafoor: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Robert Kinobe:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Funding acquisition, Data curation, Conceptualization. **Carla C.M. Chen:** Writing – review & editing, Supervision. **Noppadol Prasetsincharoen:** Visualization, Validation, Supervision, Methodology, Data curation. **Poommate Chomchat:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Nareerat Sangkachai:** Writing – review & editing, Resources, Methodology. **Orachun Hayakijkosol:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2025.107466.

Data availability

Data will be made available on request.

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