Aquatic Mammals 2023, *49*(4), 395-405, DOI 10.1578/AM.49.4.2023.395

Identification of Antimicrobial Resistance in Faecal Microbes from Wild Dugongs (*Dugong dugon*)

Alexandra M. McGowan,[1] Jennifer M. Seddon,[1] Janet M. Lanyon,[2] Nicholas Clark,[1] and Justine S. Gibson[1]

[1]*School of Veterinary Science, The University of Queensland, Gatton, QLD 4343, Australia*
E-mail: gibson.j@uq.edu.au
[2]*School of Biological Sciences, The University of Queensland, St Lucia, QLD 4072, Australia*

Abstract

Estuarine and coastal waters are areas of potential concern for antimicrobial resistance because of the discharge of wastewater from sewage treatment plants and the run-off from urban and agricultural lands. Herein, we evaluate the antimicrobial resistance profiles in bacteria from dugongs (*Dugong dugon*), mammals that inhabit and feed in shallow coastal regions and, thus, are vulnerable to encountering water and sediment contaminated by human activities. Bacterial isolates were cultured from fresh faeces of four wild dugongs, as well as from one sediment sample from a dugong foraging ground in Queensland, Australia. Ten bacterial isolates underwent phenotypic antimicrobial susceptibility testing using disc diffusion and minimum inhibitory concentration testing, and genotypic resistance and virulence gene identification through whole genome sequencing. Four *Staphylococcus warneri* isolates and one *Bacillus cereus* isolate from dugong faeces were resistant to penicillin, with two *S. warneri* isolates also displaying resistance to trimethoprim. Four *Escherichia coli* isolates were all resistant to ampicillin. Resistance genes, including *fosB*, *BcII*, *dfrC*, *blaZ*, and *mdfA*, were identified in the isolates cultured from dugong faeces with two virulence genes (*gad* and *lpfA*) identified in all *E. coli* isolates. *Lysinibacillus sphaericus* cultured from the marine sediment and *B. cereus* from dugong faeces displayed phenotypic multidrug resistance (across categories of non-extended spectrum cephalosporins, penicillins and beta-lactamase inhibitors, and clindamycin; and for *L. sphaericus*, phosphonic acids). These results demonstrate the role that dugongs can play as a sentinel species for antimicrobial resistance in the coastal waters across their range, which includes both disturbed urban and rural regions.

Key Words: dugong, *Dugong dugon*, sirenian, antibiotic, antimicrobial resistance, AMR, bacteria, faeces

Introduction

Environmental contamination and the reservoir of antimicrobial resistance (AMR) within the microbiome of wildlife are important components of the World Health Organization's global *One Health* approach to the problem of AMR (Hernando-Amado et al., 2019; White & Hughes, 2019; Torres et al., 2020). In addition to human and veterinary therapeutic applications, antimicrobial drugs have been used in livestock production to promote growth (Kummerer, 2009; Carvalho & Santos, 2016), applied to fruit and vegetable crops to control disease, and administered to commercial aquaculture stock as prophylactic agents (Kummerer, 2009; Gaw et al., 2016). Mutations leading to phenotypic resistance to antimicrobial drugs are likely to arise and be harboured in many bacterial species associated with domestic and wild animals. Selective pressure through exposure to antimicrobials then contributes to an increase in the frequency of resistance alleles and the proportion of resistant bacteria (Levy & Marshall, 2004). In addition, horizontal gene transfer of AMR genes (Hawkey & Jones, 2009) amplifies the spread of AMR in the environment. Increasingly, AMR is being identified in wildlife species for which there has been no direct therapeutic antimicrobial administration (reviewed by Ramey & Ahlstrom, 2020).

The role of environmental contamination in the increased incidence of AMR is difficult to establish. There is a tendency towards greater prevalence of resistance in wildlife when they are associated with humans, living in anthropogenic-affected habitats, and/or feeding on anthropogenic-influenced diets such as fauna feeding at landfill sites or on livestock carcasses (Ramey &

Ahlstrom, 2020). For example, *Escherichia coli*, which has a resistance to antimicrobials used in human medicine, was more prevalent in wild birds and mammals at a sewage treatment works in the United Kingdom than at two sites adjacent to farms, although there were no clear patterns of resistance prevalence with wildlife host taxonomy, spatial location, or season (Swift et al., 2019). In contrast, lower levels of AMR have been identified in pristine environments such as remote regions of Alaska (Atterby et al., 2016; but see Bhullar et al., 2012).

Marine and estuarine coastal waters are important areas for monitoring the impact of anthropogenic effects on the incidence of AMR in aquatic environments. The prevalence of antimicrobials and AMR genes in coastal waters has been linked to other indicators of pollution. A recent study comparing estuarine sediments close to two cities in China found that greater abundance of AMR genes and mobile genetic elements were associated with increased particle size in the water and, to a lesser extent, with lower salinity and pH, and higher total carbon content of the sediment (Lu et al., 2021). A systematic review of 145 peer-reviewed studies by Zheng et al. (2021) identified that antimicrobial concentrations in water and sediment were associated with organic pollutants, heavy metals, and nutrient levels, and were higher in coastal waters of low- and middle-income countries. Antimicrobials and AMR genes enter estuarine and coastal waters through riverine systems, including via agricultural and horticultural run-off (Kummerer, 2001; Carvalho & Santos, 2016), discharge from wastewater treatment plants (Stewart et al., 2014), sewage, and effluent from nearshore aquaculture farms (Gaw et al., 2016). Antimicrobials for human and veterinary use are incompletely metabolised with between 30 to 90% of administered antibiotics excreted in either urine or faeces, and sewage treatment plants are unable to completely remove these prior to discharge (Costanzo et al., 2005; Kummerer, 2009; Gaw et al., 2016). However, the relationship between resistance and anthropogenic effects is complex: AMR genes may vary by latitude, possibly reflecting temperature effects on bacterial survival, but otherwise show high spatial heterogeneity (Zheng et al., 2021).

Given the influence of anthropogenic effects on AMR in the environment, it is not surprising that AMR microbes have been cultured from a range of marine wildlife species (Foti et al., 2009; Kummerer, 2009; Rose et al., 2009; Wallace et al., 2013; Prichula et al., 2016). These studies include identifying AMR in up to 44% of stranded pinnipeds from the Northwest Atlantic (Wallace et al., 2013), from Australian sea lions (*Neophoca cinerea*) in southern Australia (Delport et al., 2015), and from common bottlenose dolphins (*Tursiops truncatus*) in Florida in the United States (Schaefer et al., 2019). Multidrug resistance has also been identified in a range of marine species, including in approximately one third of Enterobacteriales isolates from green sea turtles (*Chelonia mydas*) in northern Australia (Ahasan et al., 2017), and for bacterial species isolated from a range of marine mammals and seabirds from the U.S.'s northeastern coast (Rose et al., 2009), the North and Baltic Seas (Gross et al., 2022), and also from freshwater Amazon River dolphins (*Inia geoffrensis*; Rocha et al., 2021). Of 27 *E. coli* isolates recovered from 148 stranded marine mammals in California in the U.S. (predominantly California sea lions [*Zalophus californianus*], northern elephant seals [*Mirounga angustirostris*], and harbor seals [*Phoca vitulina*]), 71% had multidrug resistant phenotypes and genotypes (Grünzweil et al., 2021).

The dugong (*Dugong dugon*) is likely to be an appropriate sentinel species to monitor AMR in anthropogenic-affected coastal landscapes because it forages in nearshore, shallow seagrass meadows. Dugongs are a common marine mammal in northern Australia, feeding near developed coastlines subjected to residential, agricultural, and/or industrial impacts (Lanyon, 2019). The bacteria of the dugong hindgut have been characterised previously through profiling of faecal or hindgut samples (Eigeland et al., 2012; Merson et al., 2014; Jesse, 2015) and show broad similarity to those of terrestrial herbivores (Eigeland et al., 2012).

The aim of the current study was to test the presence of phenotypic AMR and AMR genes by sequencing bacteria cultured from the fresh faeces of wild dugongs. The presence of AMR was evaluated by disc diffusion and minimum inhibitory concentration (MIC) testing methods, and the identification of AMR genes through whole genome sequencing. Dugongs were sampled from two sites in coastal Queensland, Australia: (1) Moreton Bay in southern Queensland, which supports a population of approximately 900 dugongs and is adjacent to the city of Brisbane (Lanyon, 2019), and (2) the Newry Region, a *Dugong Protected Area* with a relatively small (~120 individuals) dugong population (Sobtzick et al., 2017), which is adjacent to an agricultural region in central Queensland, approximately 850 km north of Moreton Bay. This study provides some baseline evidence for the presence of AMR for two coastal regions that are predicted to be impacted by anthropogenic use of antimicrobial therapeutics.

Methods

Sampling

This study of AMR used 10 bacterial isolates (4 *E. coli*, 4 *Staphylococcus warneri*, 1 *Bacillus cereus*, and 1 *Lysinibacillus sphaericus*) obtained from four dugong faecal samples and one marine sediment sample collected for another study (McGowan, 2019; McGowan et al., in press).

Faecal samples were collected from four live wild dugongs. For two dugongs in the Eastern Banks region of Moreton Bay, southeast Queensland (27.3º S, 153.3º E), ~5 g of freshly voided faeces were collected while animals were lifted out of the water for health assessment (Lanyon et al., 2010). For the Newry Region (20.8º S, 148.9º E), two floating faecal stools were collected and an aliquot was taken from the core of each sample. These samples were in two different feeding areas and, thus, were assumed to be from two different dugongs. In addition, marine sediment was sampled by van Veen grab from a 1.5-m deep dugong foraging area in the Newry Region. Subsamples of ~5 g from each faecal and sediment sample were stored in 20% glycerol at -80ºC.

Bacterial Culture of Isolates

An aliquot of each faecal sample underwent pre-enrichment and overnight incubation in buffered peptone water (BPW) or EC Broth (4 ml, rotating at 37°C overnight; ThermoFisher Scientific, Scoresby, Victoria, Australia), followed by culture on Brilliance™ *E. coli* selective medium (ThermoFisher Scientific), on Columbia CNA medium (ThermoFisher Scientific), and/or on Sheep Blood Agar Columbia (SBA; ThermoFisher Scientific). The marine sediment sample was cultured directly on SBA plates. All cultures were incubated aerobically overnight at 37°C. DNA was extracted by heating colonies in 100 µl of ultrapure water at 100°C for 2 min. Colonies phenotypically resembling *E. coli* were confirmed by PCR using primers uspA-1 and uspA-2 (Chen & Griffiths, 1998) followed by Sanger sequencing. Other colonies were identified by PCR of a partial 16S ribosomal RNA gene using primers 16S-27F and 16S-1492R (Tsukinowa et al., 2008) followed by Sanger sequencing. For long-term storage, the isolates were placed in Brain Heart Infusion (BHI) with 20% glycerol at -80°C.

Antimicrobial Susceptibility Testing

The stored isolates were thawed and recultured on SBA, and then Kirby-Bauer disc diffusion assays (Bauer et al., 1966) were performed to determine sensitivity to antimicrobials, using the following 16 antimicrobials: amoxicillin-clavulanic acid (20/10 µg), ampicillin (10 µg), cepftazadime (30 µg), cephalothin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), enrofloxacin (5 µg), erythromycin (15 µg), fosfomycin (50 µg), gentamicin (10 µg), penicillin (10 units), sulphonamide (300 µg), tetracycline (30 µg), ticarcillin-clavulanic acid (75/10 µg), trimethoprim (5 µg), and trimethoprim-sulphamethoxazole (1.25/23.7 µg) (all from ThermoFisher Scientific, Waltham, MA, USA) following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018a, 2018b). Quality control organisms used were *Staphylococcus aureus* ATCC 2921 and *E. coli* ATCC 25922. Antimicrobial susceptibility was determined by measuring zones of inhibition and comparing against standards set by the CLSI (2018b). There are no CLSI breakpoints available for fosfomycin, so a diameter of > 32 mm was considered "sensitive" and a 0 mm diameter was considered "resistant."

Additionally, AMR was assessed using the MIC method, which was performed in duplicate for isolates that were identified as "intermediate" or "resistant" from disc diffusion testing, or for isolates that had resistant genes identified (where antimicrobials were available; CLSI, 2018a). The antimicrobials tested using MIC were amoxicillin-clavulanic acid, ampicillin, cephalothin, cephazolin, clindamycin, and penicillin (Sigma-Aldrich, Castle Hill, New South Wales, Australia). Quality control organisms used were *S. aureus* ATCC 29213 and *E. coli* ATCC 25922. CLSI guidelines (CLSI, 2018b) for bacteria isolated from animals were used for interpretative breakpoints for MICs as there were none specifically available for dugongs. *Staphylococcus* spp. breakpoints were used for *L. sphaericus* and *B. cereus* spp.

The tested antimicrobials represent ten different antimicrobial classes: penicillins, beta lactams, fluoroquinolone, cephalosporins, aminoglycosides, lincosamides, tetracyclines, trimethoprim-sulfonamides, macrolides, and fosfomycin (phosphonic acid), and 11 antimicrobial categories (Table 1). We adopted Magiorakos et al.'s (2012) definition of multidrug resistance described for *E. coli* of non-susceptibility in one or more agents in three or more antimicrobial categories.

Whole Genome Sequencing

The 10 bacterial isolates were submitted for whole genome sequencing (WGS) to assess the presence of AMR (all isolates), virulence genes (*E. coli* only), and for multilocus sequence typing (MLST; *E. coli* only). Isolates frozen in BHI and glycerol were recultured onto SBA medium, and DNA was extracted using the QIAamp DNA mini kit from QIAGEN (Hilden, Germany) following manufacturer's instructions for bacteria. DNA quality was assessed using the Qubit fluorometer (ThermoFisher Scientific), and it was submitted

Table 1. Location, source, species, and antimicrobial resistance (AMR) pattern of 10 bacterial isolates cultured from wild dugong (*Dugong dugon*) faeces ($n = 9$ isolates) and marine sediment ($n = 1$ isolate). Results for each antimicrobial (category in brackets) indicate phenotypic resistance status based on disc diffusion: R = resistant, I = intermediate, and S = susceptible. Zone of inhibition in mm shown in parentheses for fosfoymcin. Minimum inhibitory concentration (MIC; μg/ml) shown in square brackets. Blank = not tested.

Isolate ID	MB15702-3	MB15703-3	MB15703-4	NW16025-2	NW16025-3	NW16031-2	NW16031-3	NW16031-6	NW16031-7	Sedi 4-4
Location	Moreton Bay	Moreton Bay	Moreton Bay	Newry	Newry	Newry	Newry	Newry	Newry	Newry
Source	Dugong A faeces	Dugong B faeces	Dugong B faeces	Dugong C faeces	Dugong C faeces	Dugong D faeces	Dugong D faeces	Dugong D faeces	Dugong D faeces	Sediment
Species	*S. warneri*	*S. warneri*	*S. warneri*	*S. warneri*	*B. cereus*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*L. sphaericus*
Ciprofloxacin (Fluoroquinolone)	S	S	S	S	S	S	S	S	S	S
Enrofloxacin (Fluoroquinolone)	S	S	S	S	S	S	S	S	S	S
Cephalothin (Non-extended cephalosporin)	S	S	S	S	I [64]	S	S	S	S	R [64]
Cephazolin (Non-extended cephalosporin)					[> 16]					[> 16]
Ceftazidime (Extended-spectrum cephalosporin)						S	S	S	S	
Amoxicillin-Clavulanic acid (Penicillins and β-lactamase inhibitors)	S	S	S	S	S [8/4]	S	S	S	S	R [8/4]
Ampicillin (Penicillins and β-lactamase inhibitors)						R [4]	R [4]	R [4]	R [4]	
Penicillin (Penicillins and β-lactamase inhibitors)	R [4]	R [4]	R [8]	R [4]	R [> 64]					R [> 64]
Ticarcillin-Clavulanic acid (Penicillins and β-lactamase inhibitors)						S	S	S	S	
Sulphonamide (Sulfonamide)	S	S	S	S						
Trimethoprim (Folate pathway inhibitors)	R	S	S	R						
Trimethoprim-Sulphamethoxazole (Folate pathway inhibitors)	S	S	S	S	S	S	S	S	S	S
Fosfomycin (Phosphonic acids)					S (32 mm)					R (0 mm)
Tetracycline (Tetracycline)	S	S	S	S	S	S	S	S	S	S
Gentamicin (Aminoglycoside)	S	S	S	S	S	S	S	S	S	S
Erythromycin (Macrolides)	S	S	S	S	S					S
Clindamycin (Lincosamides)	S	S	S	S	I [1]					I [1]

to the Ramaciotti Centre for Genomics (Sydney, Australia) for library preparation (Nextera XT, Illumina), sequencing (Illumina MiSeq 250bp paired-end run), and quality control.

Data Analysis

Bioinformatics procedures were conducted in *Geneious*, Version r9 (https://www.geneious.com). All reads were trimmed to filter out low quality reads with an error probability limit of 0.1 and at least 20 bp at the 5' end and 30 bp at 3' end trimmed. Genomes were assembled against the following reference genomes downloaded from GenBank using *Geneious* alignment with default parameters: CP026085.1 for *E. coli* isolates, CP003668.1 for *S. warneri* isolates, CP016316.1 for *B. cereus,* and CP015224.1 for *L. sphaericus*.

Consensus sequences were uploaded to *CARD* (http://arpcard.mcmaster.ca) databases to identify AMR genes and to *ResFinder* (threshold of 80% identity; http://cge.cbs.dtu.dk/services/ResFinder) to identify AMR genes for all isolates, and chromosomal point mutations for *E. coli* sequences were uploaded to *VirulenceFinder* (threshold of 90% identity; https://cge.cbs.dtu.dk/services/VirulenceFinder) to identify virulence genes. *VirulenceFinder* does not have an option to scan for virulence genes for the other bacterial species. *E. coli* sequences were also submitted to *EnteroBase* (http://enterobase.warwick.ac.uk) to identify the MLST based on seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA*, and *recA*). These genes were downloaded for *E. coli* sequence types within the A, B1, B2, D, A×B1, and ABD phylogenetic lineages and aligned with the particular sequence types identified from the *E. coli* WGS in this study. The concatenated sequences of housekeeping genes for each sequence type were aligned using *MEGA7*, Version 7.0.26 (https://www.megasoftware.net), and a maximum likelihood phylogenetic tree was constructed using the Tamurei-Nei distance model with 1,000 bootstraps. Average nucleotide identity was calculated between the *E. coli* WGS using http://enve-omics.ce.gatech.edu/ani/index following Goris et al. (2007) to determine the similarity of isolates.

Results

Sampling, Culture, and Antimicrobial Susceptibility Testing

Collection location, source, bacterial species identity, and antimicrobial susceptibility testing results for all tested isolates from dugong faeces or marine sediment are summarised in Table 1. The four *S. warneri* isolates cultured from dugong faecal samples were resistant to penicillin, and two isolates were also resistant to trimethoprim based on disc diffusion susceptibility testing. All *E. coli* isolates were resistant to ampicillin only. The *B. cereus* isolate was resistant to penicillin and had intermediate susceptibility to cephalothin and clindamycin. The marine sediment bacterial isolate was resistant to amoxicillin-clavulanic acid, cephalothin, fosfomycin, and penicillin and had intermediate susceptibility to clindamycin. MIC testing on isolates using antimicrobials found to be "resistant" or "intermediate" from disc diffusion testing indicated that all bacterial species were resistant to all antimicrobials tested, except for clindamycin for which *B. cereus* and *L. sphaericus* isolates had intermediate susceptibility (Table 1).

Whole Genome Sequencing

A total of 47,790,042 reads were obtained from the *Illumina MiSeq* sequencing for the 10 bacterial isolates with a yield of 10.49 Gigabase pairs (GBP) and 97.4% passing quality control. The number of reads for each isolate and the percentage of reads assembled to make a single contig using the relevant reference sequence are presented in Table 2.

Submission of the *E. coli* WGS to the *EnteroBase* database identified all four *E. coli* isolates as belonging to the sequence type ST196. The maximum likelihood phylogenetic tree constructed in *MEGA7* showed that ST196 grouped most closely with sequence types belonging to the A×B1 *E. coli* clade (Figure 1). Average nucleotide identity between all *E. coli* WGS cultured from a single dugong faecal sample indicated very high similarity (> 99.9%).

Details for several resistance genes that were identified in the WGS through comparisons with previously published gene sequences are presented in Table 3. For the *B. cereus* isolate, the *CARD* database identified the *BcII* gene (91.37% identity), which confers cephalosporin resistance as consistent with the intermediate phenotypic resistance identified. Genes that confer resistance to fosfomycin were identified in *B. cereus* in comparisons with both the *ResFinder* and the *CARD* databases (87.41 to 88.41% identity; Table 3), although phenotypic resistance was not identified. *S. warneri* sequences from MB15702-3 and NW16025-2 (both 100% identity) had a trimethoprim resistance gene, which was consistent with phenotypic resistance. For *S. warneri*, isolate MB15703-4 had beta-lactamase resistance according to the *CARD* database, although all *S. warneri* showed phenotypic resistance to penicillin but not amoxicillin-clavulanic acid. In the four *E. coli* isolates, the *ResFinder* database identified macrolide resistance (*mdf(A)* gene; 98.78% identity;

Table 2. Summary of sequence reads and alignment to reference sequence information of post quality control whole genome sequencing (WGS) to reference sequences. %Q30 is the percentage of bases > Q30.

Sample ID-Isolate ID	Species	Number of reads	Mean sequence length of all reads	%Q30	% of reads assembled to form contig	Reference GenBank sequence length
Sedi 4-4	*Lysinibacillus sphaericus*	3,309,622	244.5 ± 24.8	89.8	90.12	4,692,801
NW16025-3	*Bacillus cereus*	5,037,580	243.8 ± 26.7	89.7	85.77	5,218,997
NW16025-2	*Staphylococcus warneri*	4,102,368	243.0 ± 28.4	91.8	84.92	2,486,042
MB15702-3	*S. warneri*	4,938,256	237.4 ± 35.4	92.0	85.26	
MB15703-3	*S. warneri*	3,460,458	243.9 ± 27.1	90.4	76.52	
MB15703-4	*S. warneri*	3,909,866	239.0 ± 34.0	93.3	78.79	
NW16031-2	*Escherichia coli*	3,441,848	244.3 ± 24.7	84.6	90.67	4,833,062
NW16031-3	*E. coli*	3,438,936	244.8 ± 24.1	83.9	90.34	
NW16031-6	*E. coli*	4,778,254	244.8 ± 24.0	83.2	90.49	
NW16031-7	*E. coli*	2,943,366	245.5 ± 25.2	82.8	90.61	

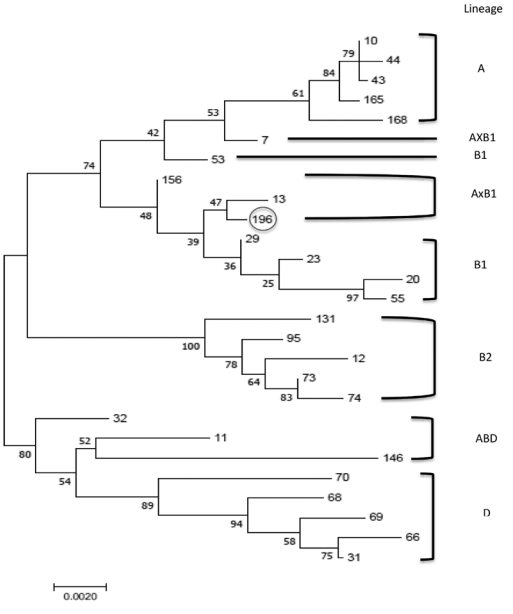

Figure 1. Phylogenetic relationships of *Escherichia coli* sequence type lineages with ST196 isolated from a Queensland dugong (*Dugong dugon*) faecal sample. Branch lengths are measured in the number of substitutions per site. ST196 is circled. A selection of subtypes from the A, B1, B2, D, A×B1, and ABD lineages were downloaded from the *EnteroBase* database.

Table 3), which was not tested phenotypically, and no genes were identified as consistent with the ampicillin resistance in the *E. coli* isolates.

The database *VirulenceFinder* identified two virulence genes in all four *E. coli* sequences: (1) the *gad* gene encodes a glutamate decarboxylase (position 2418301..2419701, accession AP010953; 100% identity), and (2) the *lpfA* gene encodes a long polar fimbriae (position 4800593..4801165, accession CP002185; 100% identity).

Hence, phenotypic multidrug resistance was identified in both *B. cereus* and *L. sphaericus*, with nonsusceptibility in at least three antimicrobial categories (non-extended spectrum cephalosporins, penicillins and beta-lactamase inhibitors, and clindamycin; and for *L. sphaericus*, phosphonic acids).

Discussion

This study identified bacteria with antimicrobial resistance (AMR) in dugong faecal samples collected from dugong foraging grounds in both urban (Moreton Bay) and agricultural (Newry Region) areas of the Queensland coast in Australia, based on phenotypic and genotypic testing. The identification of AMR in urban-associated coastal regions is likely to relate to contamination, with resistant microbes from humans or animals washing into the rivers and sea. Moreton Bay is a semi-enclosed, shallow embayment adjacent to the capital city of Brisbane, Queensland, Australia, that receives the outflow of the major Brisbane

Table 3. Resistance genes identified in the WGS of bacteria cultured from Queensland dugong faecal samples. HSP is the length of the alignment between the best matching resistance gene and the corresponding sequence in the WGS. N/A = not tested.

Sample ID-Isolate ID	Species	Database	Drug class	Gene	Position in contig	Query/HSP length	% identity	Accession #	Phenotypic resistance
NW16025-3	*Bacillus cereus*	*ResFinder*	Fosfomycin	*FosB1*	1981449..1981861	417/413	87.41	CP001903	No
NW16025-3	*B. cereus*	*CARD*	Fosfomycin	*FosB*			88.41	3000172	No
NW16025-3	*B. cereus*	*CARD*	Cephalosporin, Penam	*BcII*			91.37	3002878	Yes
MB15702-3	*Staphylococcus warneri*	*CARD*	Diaminopyrimidine antibiotic	*dfrC*			100.00	3002865	Yes
MB15703-4	*S. warneri*	*CARD*	Penam	*blaZ*			95.00	3000621	Yes
NW16025-2	*S. warneri*	*CARD*	Diaminopyrimidine antibiotic	*dfrC*			100.00	3002865	Yes
NW16031-2	*Escherichia coli*	*ResFinder*	Macrolide	*mdf(A)*	3145966..3147198	1,233/1,233	98.78	Y08743	N/A
NW16031-3	*E. coli*	*ResFinder*	Macrolide	*mdf(A)*	3145966..3147198	1,233/1,233	98.78	Y08743	N/A
NW16031-6	*E. coli*	*ResFinder*	Macrolide	*mdf(A)*	3145965..3147197	1,233/1,233	98.78	Y08743	N/A
NW16031-7	*E. coli*	*ResFinder*	Macrolide	*mdf(A)*	3145938..3147170	1,233/1,233	98.78	Y08743	N/A

River and smaller rivers that traverse the urban areas of the city and surrounding regions. The river outflow and run-off are impacted by a high summer rainfall and occasional subtropical storms, cyclones, and flooding events (Lanyon, 2019). The bay also carries significant commercial and recreational vessel traffic. Dugongs foraging on the Eastern Banks region of Moreton Bay (~10 km offshore) have shown immunological evidence of exposure to *Toxoplasma gondii* and *Neospora caninum*, both parasitic pathogens with terrestrial life cycles (Wong et al., 2019). The exposure profiles of these pathogens were most pronounced immediately after severe weather events and associated coastal flooding, and this may also be the case with antimicrobial or resistance gene contaminants. Modelling of pollutant movements within Moreton Bay (Suara et al., 2020) suggests that most debris is sourced from the land, not from the sea; only 4 to 18% of released tracer particles escaped the bay to the ocean after 282 h. This modelling indicates that there is likely to be some retention of debris contaminated with antimicrobial drugs or resistant microbes within Moreton Bay.

The three bacterial species isolated from dugong faecal samples all displayed phenotypic AMR. Disc diffusion susceptibility identified resistance to at least one antimicrobial for all bacterial isolates. All *S. warneri* isolates were resistant to penicillin, while all *E. coli* isolates, which the average nucleotide identity indicated were clonal, were resistant to ampicillin. Resistance suggested by disc diffusion testing was confirmed with MICs, with the additional identification of resistance to amoxicillin-clavulanic acid in *B. cereus* only by MICs. Of particular concern, the *L. sphaericus* isolate from marine sediment (Newry Region) had multidrug resistance, with nonsusceptibility across four of the antimicrobial categories tested.

Genotypic resistance based on analysis of the whole genome sequences was consistent with some, but not all, measures of phenotypic resistance. The genome of two of the *S. warneri* isolates (MB15702-3 and NW16025-2) contained the trimethoprim resistance gene (*dfrC*) using the *CARD* database, and both displayed phenotypic resistance. Although all *S. warneri* showed phenotypic resistance to penicillin, the beta-lactamase resistance gene *blaZ* was identified only in the MB15703-4. The resistance mechanism for this bacterial species against penicillin has not previously been identified, suggesting possible inherent or alternative resistance mechanisms. The *fosb1* gene that encodes for fosfomycin resistance was identified by both the *ResFinder* and *CARD* databases in the *B. cereus* isolate, but phenotypic resistance was not detected utilising disc diffusion testing based on the breakpoints used. MIC was not estimated due to the unavailability of this antimicrobial. The resistance guidelines used in this study (CLSI, 2018a, 2018b) were developed mainly for companion and production animals. Further refinement of the resistance breakpoint for fosfomycin for the dugong *B. cereus* isolates and the development of specific guidelines for dugongs would be beneficial.

The findings in this study are broadly similar to those reported by other studies investigating AMR in marine mammals. For example, *Staphylococcus* spp. isolated from live- and dead-stranded sea otters (*Enhydra lutris*) from California and Alaska had either intermediate resistance or were above the resistance threshold for penicillin based on MIC testing, as were *E. coli* isolates tested against ampicillin (Brownstein et al., 2011). Multidrug resistance has been recorded for bacterial species isolated from wild bottlenose dolphins in the southeastern U.S., with 48% of dolphins harbouring bacteria resistant to more than one antibiotic (Stewart et al., 2014). In a region ~300 km north of the Newry Region sampled in this study, wild green sea turtles foraging in Cleveland Bay (Townsville) were resistant to multiple antibiotics, with the highest frequency of resistance against the beta-lactam class antimicrobials (Ahasan et al., 2017). These initial studies indicate that AMR in marine species is likely to be widespread, at least in coastal areas experiencing anthropogenic impacts.

The multi-locus sequence typing of the *E. coli* WGS identified it as belonging to a previously described sequence type, ST196, which grouped within the A×B1 lineage on phylogenetic analysis. This sequence type has previously been identified in association with terrestrial species or environments, including production and companion animals; humans; and wildlife from Australia, the U.S., Europe, Africa, Asia, and Europe (Fischer et al., 2014, 2017; *EnteroBase* database). *E. coli* isolated from marine mammals in California included the different sequence types ST38 and ST648, and the pandemic clone of public health concern, ST167 (Grünzweil et al., 2021).

Virulence genes were also identified in the four *E. coli* WGS. The *gad* and *lpfA* genes were identified by the *VirulenceFinder* database with 100% identity. The enzyme glutamate decarboxylase (GAD) is found in all *E. coli* strains. Its function is to protect cells in acidic environments (Bergholz et al., 2007). The long polar fimbria protein A (*lpfA*) gene has been found in clinical and commensal isolates from humans and cattle and is associated with cell adhesion (Toma et al., 2006; Blum & Leitner, 2013; Kidsley et al., 2018).

Dugongs could act as a coastal sentinel species for AMR in bacteria that are clinically relevant to

mammalian species, including humans. This study provides an initial indication that AMR, including multidrug resistance, is present in bacteria from dugongs and their east coast Australian habitat. Further longitudinal research, including genomic sequencing, is required to understand if wildlife species harbouring resistant bacteria are acting as amplifiers or disseminators of resistance, to pinpoint the directionality of AMR spread, and to provide more definitive information on the source attributions of resistance (Ramey & Ahlstrom, 2020).

Acknowledgments

Many thanks to the people who helped collect dugong faecal samples, including members of The University of Queensland (UQ) Dugong Research Team, especially Helen Sneath and Rob Slade. Thank you to Rochelle Price, Tina Maguire, and Charmaine Lubke from the UQ School of Veterinary Science Laboratory Services for their assistance in the microbiology lab, including antimicrobial resistance testing of bacterial isolates. Thank you to Sean Corley and Deanne Waine from the UQ Animal Genetics Laboratory for their assistance in the lab. Dugongs were sampled under The University of Queensland Animal Ethics Permit SBS/181/18, Scientific Purposes Permit WISP14654414, Moreton Bay Marine Parks Permit MPP18-001119, and Great Barrier Reef Marine Park Permit G14/36987.1. Funding was provided by Sea World Research and Rescue Foundation Inc. (SWR/1/2015; SWR/6/2016) and the Winifred Violet Scott Foundation (WVS/1/2015).

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