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Whole-genome analysis of extraintestinal pathogenic *Escherichia coli* (ExPEC) MDR ST73 and ST127 isolated from endangered southern resident killer whales (*Orcinus orca*)

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Background: Limited studies have investigated the microbial diversity of wild marine mammals.

Objectives: This study characterized *Escherichia coli* isolates collected from fresh faecal samples of endangered southern resident killer whales (*Orcinus orca*) located by detection dogs.

Methods: WGS of each strain was done to determine ST (using MLST), clonotype (C:H), antimicrobial resistance and virulence profile. Conjugation experiments were done to determine the mobility of the *tet*(B) tetracycline resistance gene.

Results: All isolates belonged to extraintestinal pathogenic *E. coli* (ExPEC) clonal lineages ST73 (8/9) and ST127 (1/9), often associated with human community-acquired urinary tract disease. Clonotyping using *fumC* and *fimH* alleles showed divergence in clonal lineages, with ST73 isolates belonging to the C24:H10 clade and the ST127 isolate belonging to C14:H2. The eight ST73 isolates carried multiple acquired antibiotic resistance genes, including *aadA1*, *sul1* and *tet*(B), encoding aminoglycoside, sulphonamide and tetracycline resistance, respectively. Conjugative transfer of the resistance gene *tet*(B) was observed for three of the eight isolates. ST127 did not carry any of these acquired resistance genes. Virulence-associated genes identified included those encoding adhesins (*iha*, *papC*, *sfaS*), toxins (*sat*, *vat*, *pic*, *hlyA*, *cnf1*), siderophores (*iutA, fyuA, iroN, ireA*), serum survival/protectins (*iss, ompT*), capsule (*kpsM*) and pathogenicity island marker (*malX*).

Conclusions: Orca whales can carry antibiotic-resistant potentially pathogenic strains of *E. coli*. Possible sources include contamination of the whale's environment and/or food. It is unknown whether these isolates cause disease in southern resident killer whales, which could contribute to the ongoing decline of this critically endangered population.

Introduction

We identified and characterized MDR extraintestinal pathogenic *Escherichia coli* (ExPEC) from fresh faecal samples collected from endangered southern resident killer whales (*Orcinus orca*). These bacteria strains are commonly associated with community-acquired urinary tract infections in humans.[1] These orcas are apex predators in the Salish Sea and are known to forage as pods along the inner waters of British Columbia and Washington State during summer months (May–October).[2] There are three pods (J, K and L) and their population has fluctuated from 98 whales in 1995 to 75 as of 11 January 2019.[2] They are listed as critically endangered by both the United States Endangered Species Act (2005) and the Canadian Species at Risk Act (SARA) (2001).[2]

A single study has analysed the respiratory bacteria from exhaled breath of several southern resident killer whales and detected potentially pathogenic and antibiotic-resistant species.[3] To date, no studies have investigated *E. coli* among these endangered whales. In the current study, we used WGS to analyse nine *E. coli* isolates collected from southern resident killer whale faecal samples to identify strain types and characterize their antibiotic resistance and virulence profiles.

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Materials and methods

Specimen collection and culture

Eleven fresh southern resident killer whale faecal samples were collected in the Salish Sea around San Juan Islands in Washington State between August and October 2013 (Figure 1a). Sample collection methods were approved by the University of Washington's Institutional Animal Care and Use Committee (IACC) under protocol 2850-08. Trained dogs were employed on boats and the canine's keen olfactory system was utilized to detect specific southern resident killer whales' faecal scent from distances farther than a nautical mile.[4] Samples were collected as part of a previous study and centrifuged into a small pellet on the boat as previously described.[4] For the current study, sterile Fisherbrand cotton swabs (Fisher Scientific, Waltham, MA, USA) were inserted into the homogenized faecal pellet and ~0.5 mL of each sample was removed and stored in 10 mL of sterile peptone water on ice. Samples were returned to the laboratory within 2–6 h of collection and vortexed for 10 s, and 0.1 mL was spread on MacConkey agar plates (Difco Laboratories, Sparks, MD, USA) unsupplemented or supplemented with antibiotics (including 25 mg/L tetracycline, 25 mg/L chloramphenicol or 25 mg/L ampicillin) and incubated at 36.5°C for 24–48 h. Nine of the 11 samples tested positive for *E. coli* and 8 of the 9 grew on Difco™ LB medium (Difco) supplemented with 25 mg/L tetracycline. Single *E. coli* isolates from different samples and plates were verified using standard biochemical tests. No *E. coli* were detected on either the ampicillin- or chloramphenicol-supplemented medium. No *E. coli* could be isolated from 74 freeze-dried frozen faecal samples stored for >1 year.

WGS, assembly and analysis

DNA extraction of the nine isolates was done using a DNeasy UltraClean® Microbial DNA Isolation Kit (Mo-Bio Laboratories, Carlsbad, CA, USA). Dual-index libraries were prepared using a Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) with 1 ng of bacterial DNA and 14 amplification cycles. The libraries were sequenced using Illumina MiSeq. Isolates 2-J28, 3-J8, 4-UK, 6-J26 and 7-J27 were sequenced on a 1×192 bp run and due to logistical reasons 1-J28, 5-L79, 8-J31 and 9-J31 were re-sequenced on a 2×300 bp run. Raw reads were trimmed using Trimmomatic for quality, *de novo* assembled using SPAdes Genome Assembler v3.11 and annotated using Prokka v1.13.[5–7] Sequence data were deposited in NCBI GenBank under project PRJNA338014 with accession numbers RQHZ00000000, RQIE00000000, RRTX00000000, RQID00000000, RQIC00000000, RQIB00000000, RQIBA00000000, RQHY00000000 and RQHX00000000 (1-J28 to 9-J31). Assembled contigs were uploaded into ResFinder and VirulenceFinder (https://cge.cbs.dtu.dk/services) to identify acquired antibiotic resistance genes and virulence genes with identity thresholds of 98% and 96%, respectively.[8,9] Virulence genes were determined using available reference literature, NCBI GenBank and Geneious software.

Sequence typing, clonotyping, SNPs and phylogenetic comparison

In silico analysis of isolates was done to determine MLST ST based on the Achtman housekeeping gene scheme. Clonotyping was done using the *fumC* and *fimH* (type 1 fimbrial adhesin) allele Weissman method.[10] Core-genome SNPs were identified using Snippy v 4.3.2 and known ExPEC isolate CFT073 (AE01475.1) was used as the reference strain for sequence alignment.[11] Phylogenetic analysis was based on SNP alignment and trees were constructed using neighbour joining (NJ), maximum likelihood (PHYML) and Bayesian (Mr Bayes) methods in Geneious Pro 9.1.5 under default settings[12] (Figure 1b).

Antimicrobial resistance and conjugation experiments

Antimicrobial resistance was assessed by Kirby–Bauer disc diffusion (Difco Laboratories, Division Becton-Dickinson) and MIC methods according to a CLSI protocol with standard *E. coli* ATCC 25922 used as the control.[13] Isolates were streaked on Mueller–Hinton agar, forming a lawn, and the following antibiotic discs were aseptically placed: chloramphenicol, cefepime, spectinomycin, ciprofloxacin, gentamicin and tetracycline (Becton-Dickinson Microbiology Systems, Franklin Lakes, NJ, USA). Plates were incubated for 18–24 h at 36.5°C. Antibiotics used for MIC testing included streptomycin, spectinomycin and sulfisoxazole. Results were recorded according to CLSI standards.[13]

To test whether the isolates carried mobile *tet*(B) resistance genes, conjugation experiments were done with the eight tetracycline-resistant isolates and with 50 mg/L rifampicin-resistant *E. coli* HB101 as a recipient strain. The mating mixture used a 1:100 ratio (donor to recipient) and mating plates were incubated at 36.5°C for 24–48 h. Transconjugants were isolated from plates and verified by growth on LB medium supplemented with 25 mg/L tetracycline and 50 mg/L rifampicin as previously described.[14]

Results

WGS and SNP analysis of southern resident killer whale *E. coli*

WGS was performed for all *E. coli* isolates. Mean genome contig size ranged from 3124 bp (1-J28) to 11974 bp (9-J31). Assembly sizes ranged from 4.8 Mb (8-J31) to 5.11 Mb (9-J31) (average of 5.0 Mb). By *in silico* MLST, all isolates belonged to ExPEC clonal lineage strain types ST73 (8/9) and ST127 (1/9). Clonotyping exhibited further divergence between clones, with all ST73 isolates belonging to the C24:H10 clonotype and the ST127 isolate belonging to the C14:H2 clonotype (Table 1). All the ST73 isolates from the current study were genetically closely related based on SNP analyses of the core genome (8–68 different SNPs), but less related to the reference strain CFT073 (>3000 SNP differences), while ST127 was distinct, with >26000 SNP differences (Figure 1b).

Genotypic and phenotypic antimicrobial resistance and virulence profile

Antibiotic resistance genes identified at >98% identity included *aadA1*, *sul1* and *tet*(B), encoding aminoglycoside, sulphonamide and tetracycline resistance, respectively, for all eight ST73 isolates. No antibiotic resistance genes were found in the ST127 isolate (Table 1). All eight ST73 isolates were resistant to tetracycline (30 mg/L) and grew on sulfisoxazole-supplemented (512 mg/L) or spectinomycin-supplemented (100 mg/L) plates, but only two isolates (3-J8 and 7-J27) showed resistance to streptomycin (50 mg/L).

Virulence factor genes were determined *in silico* using reference literature, BLAST and Geneious software at >96% identity. Sixteen different virulence factor genes were identified, including those encoding adhesins (*iha*, *papC*, *sfaS*), toxins (*sat*, *vat*, *pic*, *hlyA*, *cnf1*), siderophores (*iutA, fyuA, iroN, ireA*), serum survival/protectins (*iss*, *ompT*), capsule (*kpsM*) and pathogenicity island marker (*malX*) (Table 1).

Mobility of tet(B)

All eight tetracycline-resistant isolates were tested for their ability to transfer the *tet*(B) gene. Three isolates (1-J28, 5-L79 and 4-UK) were shown to transfer the *tet*(B) gene to recipient *E. coli* HB101 at

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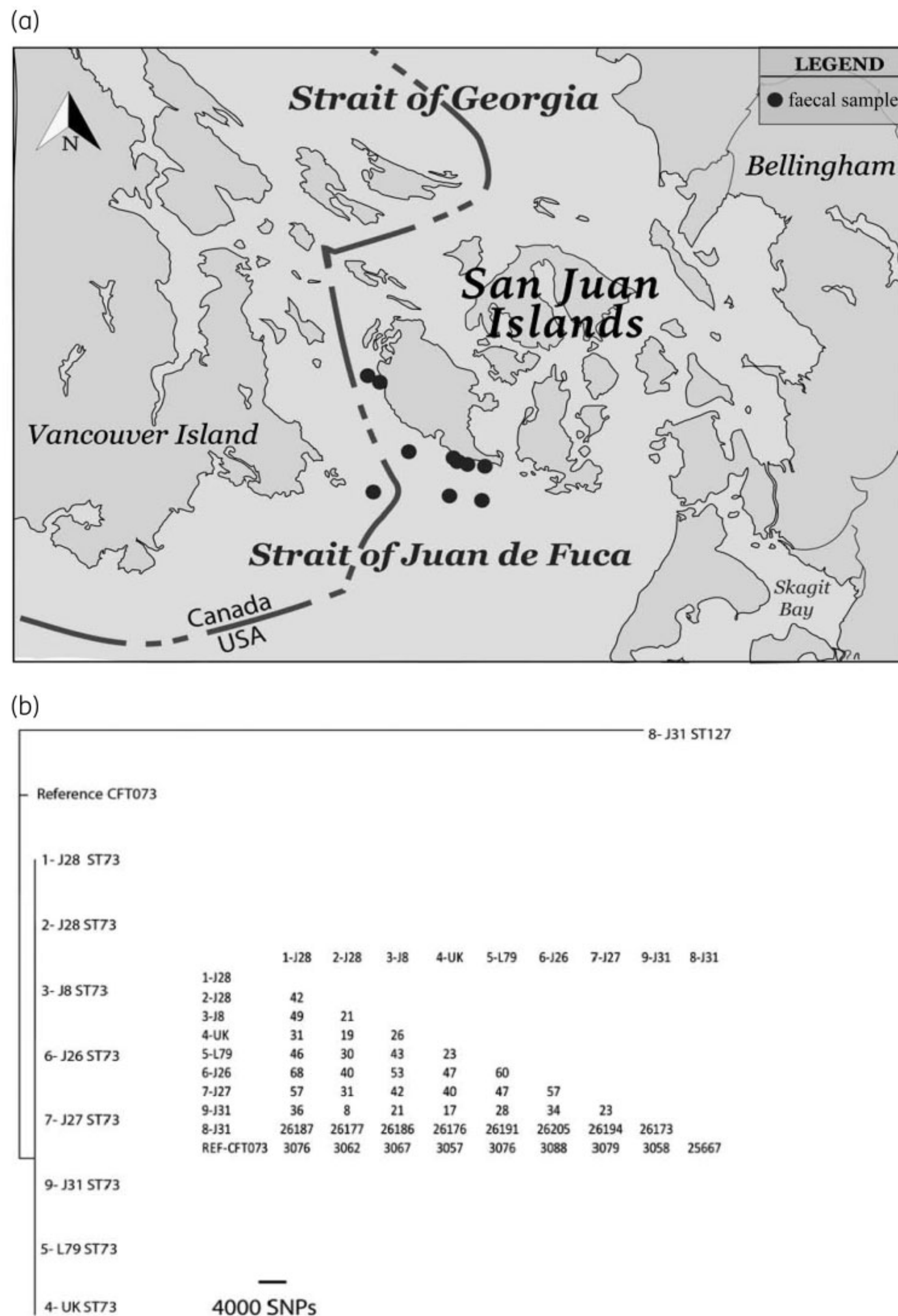

	1-J28	2-J28	3-J8	4-UK	5-L79	6-J26	7-J27	9-J31	8-J31
1-J28									
2-J28	42								
3-J8	49	21							
4-UK	31	19	26						
5-L79	46	30	43	23					
6-J26	68	40	53	47	60				
7-J27	57	31	42	40	47	57			
9-J31	36	8	21	17	28	34	23		
8-J31	26187	26177	26186	26176	26191	26205	26194	26173	
REF-CFT073	3076	3062	3067	3057	3076	3088	3079	3058	25667

Figure 1. (a) Geographical representation of sample collection area. Filled circles indicate where faecal samples were collected in the Salish Sea (created using Adobe Illustrator). (b) Phylogenetic analyses of core-genome pairwise SNPs of *E. coli* ST73 and ST127 with CFT073 as the reference strain. Matrix of numbers of SNP differences.

a frequency of 5.9×10^{-5}, 1.1×10^{-8} and 9.0×10^{-8}, respectively, suggesting a plasmid location since *tet* genes are not normally found on integrons.[15] The other five did not transfer at measurable frequencies.

Discussion

In this study, ExPEC clonal strains ST73 and ST127 were characterized using WGS and SNP analysis (Table 1 and Figure 1). WGS was used to characterize and identify antibiotic resistance genes, while disc diffusion and MIC tests were performed for phenotypic

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Table 1. *E. coli* isolate characterization, antibiotic resistance and virulence gene profile

Isolate	ST[a]	Clonotype[b]	Antibiotic resistance[c]	Virulence gene profile[d]
1-J28	ST73	C24:H10	*aadA1*, *sul1*, *tet*(B)	*iha*, *papC*, *sfaS*, *sat*, *hlyA*, *iutA*, *fyuA*, *ompT*, *iss*, *kpsM*, *malX*
2-J28	ST73	C24:H10	*aadA1*, *sul1*, *tet*(B)	*iha*, *papC*, *sfaS*, *sat*, *vat*, *pic*, *iroN*, *ireA*, *iutA*, *fyuA*, *iss*, *ompT*, *kpsM*, *malX*
3-J8	ST73	C24:H10	*aadA1*, *sul1*, *tet*(B)	*iha*, *papC*, *sfaS*, *sat*, *vat*, *pic*, *iroN*, *ireA*, *iutA*, *fyuA*, *iss*, *kpsM*, *malX*
4-UK[e]	ST73	C24:H10	*aadA1*, *sul1*, *tet*(B)	*iha*, *papC*, *sfaS*, *sat*, *vat*, *pic*, *iroN*, *ireA*, *iutA*, *fyuA*, *iss*, *kpsM*, *malX*
5-L79	ST73	C24:H10	*aadA1*, *sul1*, *tet*(B)	*iha*, *papC*, *sfaS*, *sat*, *pic*, *iroN*, *ireA*, *iutA*, *fyuA*, *kpsM*, *malX*
6-J26	ST73	C24:H10	*aadA1*, *sul1*, *tet*(B)	*iha*, *papC*, *sfaS*, *sat*, *pic*, *iroN*, *iutA*, *fyuA*, *iss*, *kpsM*, *malX*
7-J27	ST73	C24:H10	*aadA1*, *sul1*, *tet*(B)	*iha*, *papC*, *sfaS*, *vat*, *pic*, *hlyA*, *iutA*, *ireA*, *fyuA*, *kpsM*, *malX*
9-J31	ST73	C24:H10	*aadA1*, *sul1*, *tet*(B)	*iha*, *sat*, *vat*, *pic*, *iroN*, *ireA*, *iutA*, *fyuA*, *iss*, *pic*, *kpsM*, *malX*
8-J31	ST127	C14:H2		*sfaS*, *cnf1*, *vat*, *iroN*, *ireA*, *fyuA*, *iss*, *ompT*, *kpsM*

[a]STs are in accordance with the Achtman scheme based on housekeeping genes.
[b]Clonotyping was done using *fumC* and *fimH* alleles (C: H).
[c]Antibiotic resistance genes (corresponding drug class): *aadA1* (aminoglycoside), *sul1* (sulphonamide) and *tet*(B) (tetracycline).
[d]Virulence factor genes encoding adhesins (*iha*, *papC*, *sfaS*), toxins (*sat*, *vat*, *pic*, *hlyA*, *cnf1*), siderophores (*iutA*, *fyuA*, *iroN*, *ireA*), serum survival/protectins (*iss*, *ompT*), capsule (*kpsM*) and pathogenic island marker (*malX*).
[e]Unknown whale; could be a transient whale with a different lifestyle from the whales in pods J, K and L.

verification. The SNP analyses of core genomes showed a high degree of genetic relatedness among the whale ST73 isolates (Figure 1). The ST73 isolates were phenotypically resistant to tetracycline and contained *tet*(B), and also showed resistance to aminoglycosides (spectinomycin and streptomycin) and sulphonamides (sulfisoxazole). Similar ExPEC clonal groups (ST73, ST127 and ST131) have been isolated in faecal samples collected from wild marine mammals (Antarctic pinnipeds).[16] The ExPEC isolates from the Antarctic study were resistant to ampicillin, cefalotin and nalidixic acid, which differed from our study, where the antibiotic resistance genes are normally associated with mobile elements.[14,15] Both studies identified similar virulence factor genes, including *fimH*, *iroN*, *cnf1*, *sfaA* and *hlyA*.[16] However, in the present study variability in carriage of these virulence factor genes was found, with some frequently associated with ExPEC plasmids (*ompT*, *iutA*, *iroN* and *iss* genes) (Table 1).[17]

Previous studies have shown that the Salish Sea is contaminated with anthropogenic pollution, including the presence of multiple antibiotic resistance genes.[18] Analyses of water samples and resident whole-body fish tissues from the region also identified numerous contaminants of emerging concern, including antibiotic residues near waste water treatment plant ('WWTP') discharge sites, as well as in resident juvenile wild Chinook salmon (*Oncorhynchus tshawytscha*). The study of Meador *et al.*[19] suggested bioaccumulation of these chemical pollutants and further hypothesized that this bioaccumulation in the trophic level of the food chain could be potentially dangerous to the southern resident killer whale population, as they are known to forage for fish, exclusively salmonids.[20]

The current study showed that southern resident killer whales carried antibiotic-resistant and potentially pathogenic strains of *E. coli*, which have likely been acquired either directly from the Salish Sea or from their salmon diet. It is unknown whether these isolates could cause disease in these endangered whales or whether they contribute to the population's ongoing decline. Clearly, further studies on the microbial diversity of these orcas should be done as part of future investigations to help with their recovery efforts.

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Transparency declarations

None to declare.

Disclaimer

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