Whole Genome analysis of extraintestinal pathogenic *Escherichia coli* (ExPEC) isolated from the endangered Southern Resident Killer Whales (SRKW; *Orcinus orca*)

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ABSTRACT

Whole Genome analysis of extraintestinal pathogenic *Escherichia coli* (ExPEC) isolated from the endangered Southern Resident Killer Whales (SRKW; Orcinus orca)

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With current evidence of increased chemical contamination and antimicrobial resistance (AMR) in environmental samples from the Salish Sea, it is important to examine its resident wild life to further investigate the detrimental effects of these anthropogenic pressures. Surveillance of antibiotic resistant bacteria in marine mammals adds to our understanding of the health of the ecosystem, as well as the spread of AMR in these susceptible environments. To date, few studies have examined the prevalence of pathogenic microbes in wild cetacean species, specifically the critically endangered Southern Resident Killer Whales (SRKW; *Orcinus orca*). This population is known for their intimate connection to the Salish Sea, and act as sentinels for its vulnerable marine ecosystem. The objective of this study was to utilize next-generation sequencing tools to characterize and analyze nine *Escherichia coli* isolates collected from fecal samples of these critically at-risk orcas.

For this study, we performed whole-genome sequencing (WGS) and *de-novo* assembly of each isolate to ascertain strain lineage by individual strain sequence type (ST), clonotype (C:H), antimicrobial resistance and virulence profile. By multi-locus sequence typing (MLST), all isolates belonged to either extraintestinal pathogenic *E. coli* (ExPEC) clonal lineage ST73 (8/9)

or ST127 (1/9), lineages often associated with human and animal urinary tract infections. Clonotyping using *fumC* and *fimH* alleles showed further divergence in these clones with ST73 isolates belonging to the C24:H10 clade, and the ST127 to C14:H2. All eight ST73 clones carried multiple AMR genes including *aadA, sul1* and *tet*(B), coding for resistance to aminoglycoside, sulfonamide, and tetracycline, respectively. Conjugative transfer of resistance *tet*(B) gene was observed for three of these nine isolates suggesting presence of mobile genetic elements. The ST127 isolate did not carry any resistance genes. Sixteen virulence-associated genes identified include: adhesins (*iha, papC, sfaS),* toxins (*sat, vat, pic, hlyA, cnf1),* siderophores *(iroN, ireA, iutA, fyuA),* serum survival/protectins (*iss, ompT),* capsule (*kpsM*), and pathogenicity island marker (*malX).*

This study helps to advance our understanding of the dissemination of antimicrobial resistant *E. coli* in the Salish Sea, and demonstrates the need for increased surveillance efforts for any microbial factors potentially impacting the health of the SRKW. Additionally, it supports the use of next generation sequencing tools for increased and high-resolution insight into the genomic landscape of indicator bacteria for endangered species and other wildlife. Antimicrobial resistance is a global and emerging threat, affecting human, animal, and environment health. This study showed that the SRKW can carry antibiotic resistant, potentially pathogenic strains of *E.coli*. Possible sources include contamination of the orca's environment and/or food. While it is unknown if these isolates cause disease in the SRKW which could contribute to the ongoing decline of this critically endangered population.

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ABBREVIATIONS AND ACRONYMS

AMR: Antimicrobial Resistance

APEC: Avian pathogenic *E. coli*

ARG: antibiotic resistance genes

CECs: contaminants of emerging concern

CDS: coding sequences

CWR: Center for Whale Research

E. coli: *Escherichia coli*

ExPEC: Extra-intestinal pathogenic *E. coli*

MDR: multidrug resistant

MLST: multi-locus sequence type

MIC: minimum inhibitory concentrations

NCBI: National Center for Biotechnology Information

NOAA: National Oceanic Atmospheric Administration

PCBs: polychlorinated biphenyls

POPs: persistent organic pollutants

SNP: single nucleotide polymorphism

SRKW: Southern Resident Killer Whale

UPEC: Uropathogenic E. coli

UTIs: Urinary Tract Infections

VF: virulence factor

VAG: virulence associated-gene

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PUBLICATION

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   More data are presented in the thesis than in this brief publication

INTRODUCTION

The Southern Resident killer whales (SRKW; *Orcinus orca*), also known as the "Orcas of the Salish Sea" are distinguished by the fact that they spend a majority of the year (Spring- Fall) traveling and foraging as multi-generational pods within the Salish Sea, along the inner waters of British Columbia and Washington State (Center for Whale Research). The clan's intimate relationship to this specific geographic location is integrated within the region's culture and identity and has been instrumental to their monitoring since the early 1970's. The clan consists of three pods (J, K, L), and its population has fluctuated from 98 whales in 1995 to 75 as of January 2019 (Center for Whale Research, 2019). Due to this sharp decline, both the United States Endangered Species Act (2005), and the Canadian Species at Risk Act (SARA) (2001) list the SRKW as endangered (CWR). Major threats to their health include: decreased prey supply, anthropogenic contaminants, and disturbance from private and commercial vessels (UW Conservation Biology, 2019). As apex predators, these cetacean creatures act as sentinels for their resident environment and are extremely valuable in assessing the overall health of the Salish Sea ecosystem.

The Puget Sound is one of the major bodies of water found within the Salish Sea. Unfortunately, for many decades it has been subjected to increased pollution from human pressure including discharge from agricultural and municipal activities, predominantly from wastewater treatment plant (WWTP) effluent. This environment has now become a hot spot for antibiotic resistant bacteria and other chemical contaminants including persistent organic pollutants (POPs). In 2010, a metagenomic profile on the prevalence of microbial organisms of its surface water samples (~5 m depth) revealed presence of antimicrobial resistance genes and mobile genetic elements across metagenomes (Port et al, 2012). Further collaborative research

by the National Oceanic Atmospheric Administration's Northwest Fisheries Science Center and the University of Washington has also identified contaminants of emerging concern (CECs) including pharmaceutical compounds and antibiotic residues (Meador et al, 2016). In addition to environmental water samples, Meador et al, (2016) also looked at toxicant spread in wild resident fish including Chinook salmon (*Oncorhynchus tshawytscha*) and found CEC's in their tissue. It is hypothesized that bioaccumulation of toxic chemicals in the trophic level of the food chain could be potentially dangerous to the SRKW population. Investigating food sources is important as these orcas are known to forage for fish, exclusively salmonids (Ford et al, 2006). One study collected SRKW fecal samples for 5 years and genetically analyzed it to gain insight into their diet composition; results showed that salmonids made up over 98% of the SRKW summer diets, specifically the Chinook salmon which accounted for 79.5% of the sequences (Ford et al, 2006).

A limited number of studies have looked at anthropogenic pollutants and their direct adverse effects on the SRKW population. One study investigated SKRW blubber biopsies to gain insight on potential concentrations of persistent organic pollutants (POPs) and its consequences. Krahn et al, (2007) showed that SRKW had elevated levels of polychlorinated biphenyls (PCBs) that exceeded thresholds for health in captive marine mammals. Additional studies on elevated POPs in SRKW population not only found similar increased levels of PCBs among all, but also discovered that the juvenile whales had higher levels of contaminants, presumably as a result of maternal transfer during lactation (Krahn et al, 2009). More recently, investigations on POPs from SRKW were done by collection of fecal samples using trained detection dogs. Lundin et al, (2016) collected 140 fecal samples and results showed patterns of POP concentrations consistent with blubber biopsies from previous studies. This non-invasive surveillance method has opened

the door for future less-invasive monitoring of the SRKW and other marine mammals that inhabit the Salish Sea. Limited research on the microbiota of wild SRKW has been initiated; one study analyzed the respiratory bacteria from exhaled breath of several SRKW. Raverty et al, (2017) detected numerous potentially pathogenic and antibiotic resistant bacteria including: *Staphylococcus epidermidis, S. aureus, Pseudomonas fluorescens,* and *Salmonella enterica*. To date however, no studies have reported the presence of *E. coli* among these endangered animals.

E. coli act as an ideal reference bacterium for the health of wild life and environments. It is considered to be an index bacterium, often found as part of the normal gut flora of mammals. Research on wild marine mammals regarding the presence of *E. coli* is sparse. One study looked at stranded Arctic pinnipeds and presence of bacteria to investigate whether isolation stressors are correlated with potential pathogenic microbial colonization. Wallace et al, (2013) found several different bacteria including *E. coli* in the pinnepeds that showed increased resistance to several antimicrobial classes; however, this study performed no further characterization of the bacteria.

Furthermore, the concept of "One Health" is important as it highlights the interrelationships of human, animal, and environmental health. Coastal environments like the Salish Sea are important settings to study the effects of anthropogenic pollution on ecosystem health and its resident wildlife. Understanding the ecology of infectious organisms in the SRKW population will not only educate us about their population dynamics but can also warn us of potential public health risks.

This study utilized next-generation sequencing tools to analyze and characterize *E. coli* isolated from fecal samples collected from the SRKW. The isolates belonged to a pathogenic lineage of extra-intestinal *E. coli* (ExPEC). Their antimicrobial resistance and virulence profiles

indicated carriage of multiple ARG's and virulence factor genes (VFs). Many of the VFs were associated with ExPEC mobile genetic elements and conjugation experiments with the *tet*(B) further demonstrated capabilities for transferring of pathogenesis between bacteria.

LITERATURE REVIEW

Extraintestinal pathogenic *E. coli* (ExPEC) ecology and relation to health

E. coli, a gram-negative bacterium found in the gut microflora of both human and animals, can often be commensal, yet highly adapted pathogenic clones have been discovered and found to be associated with different disease outcomes (Riley, 2014). The differences between strains of this bacterium can be highly variable; however, disease-causing *E. coli* fall into two different categories: those that cause illness in the gastrointestinal tract, and those that cause extraintestinal pathology (ExPEC) (Kaper et al, 2004). ExPEC are distinguished in their ability to inhabit niches outside of the gut and cause different types of disease, including urinary tract infections (UTIs), and septicemia (Russo & Johnson, 2002). Additionally, several clones of this type of pathogenic *E. coli* have been found to cause disease in both humans and animals with some strains sharing common genetic backgrounds, suggesting zoonotic potential(Johnson et al, 2008, Clermont et al, 2011, Belanger et al, 2011). According to Russo and Johnson (2005), uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC), and sepsis-causing *E. coli* (SEPEC) are collectively grouped as ExPEC. Additionally, avian pathogenic *E. coli* (APEC), a known etiologic agent of disease in birds, has also been connected to human ExPEC strains due to similarities in virulence profiles (Kaper et al, 2004).

One investigator, Riley (2014) examined the widespread geographical dissemination of different human ExPEC pandemic clones from nine studies and found ST73 as one of the major strain lineages associated with hospital and community-acquired urinary tract and bloodstream infections (Riley, 2014). For his study, the author utilized MLST as the typing method to separate the study isolates into separate clonal groups. Not only did he find ST73 as one of the

predominant ST types, but he also detected the presence of ST127 strains, albeit in lower frequencies (Riley, 2014).

Pathogenesis & Virulence factors for EXPEC

ExPEC can cause different disease pathologies outside of the intestine; however, its most common site of infection is the urinary tract (UPEC) (Johnson and Russo, 2002). UPEC clones have unique phenotypic profiles that cause UTI's, including pathogenicity islands containing virulence genes different from the non-pathogenic commensal strains (Kaper et al, 2004, Blum-Oehler et al, 2000). ExPEC utilizes many virulence factors (VF) to invade the body; these range from adhesins and invasins, to toxins and iron acquisition siderophores. Additionally, some ExPEC VF are found to be frequently encoded on mobile genetic elements including plasmids, integrons, and transposons which allow them to mobilize into other strains to create novel combinations (Kaper et al, 2004). Possession of these genes is not only important for ExPEC strains to establish themselves and overcome their host-defenses, but also necessary to cause a successful infection.

Adhesins

ExPEC need to carry a combination of specific VFs which will allow them to successfully colonize and cause infection in organs outside of the intestinal tract (Johnson and Russo, 2002). Adhesion VF is required for successful extra-intestinal infections (Kaper et al, 2004). ExPEC can carry different types of adhesins that produce proteins with distinct morphological structures including fimbriae (also pili), and adhesion siderophores (*iha)* distinct from common flagella [not associated with pathogenicity] (Kaper et al, 2004). According to Johnson (1991), two common UPEC adhesives include: type 1 fimbriae (*fimH*) and P fimbriae. Type 1 fimbriae help the bacteria adhere to the urinary tract by expressing an adhesive tip

protein: *fimH*. Other studies have also shown/supported that these type 1 fimbriae are necessary VFs for ExPEC infection as they help to facilitate the persistence of urinary tract-infections (Mobley et al, 1987). The P fimbriae are composed of a cluster of different attachment-associated proteins including *papG* and *papC* (Lund et al, 1988). Lane et al (2007) highlighted the role of P fimbriae for adherence in persistence of UPEC infection in mammalian kidneys. Additional studies have shown that the *sfaS* gene codes for an adhesion protein commonly associated with ExPEC isolates. This protein acts to form the S fimbriae, which helps to attach the bacteria to epithelial and endothelial cells of the urinary tract/kidney (Mulvey et al, 2002; Bien et al, 2012).

Toxins & Siderophores

In order to induce pathogenesis and infection in the host, ExPEC must also carry different toxin producing genes. Toxins include cytotoxic necrotizing factors (*cnf1*), autotransporters (*sat, vat, pic),* and pore-forming toxins (*hlyA),* (Ulett et al, 2013). Additionally, it is also crucial for ExPEC strains to scavenge for iron to successfully survive and cause infection. Siderophores important for ExPEC survival outside the intestines include *iroN, fyuA, ireA, iutA* (Kaper et al, 2004). Additionally, Magistro et al (2015) found that the *iroN* gene (known for its iron acquisition system) is also important for putative action as it influences biofilm formation.

Pathogenesis in humans

In humans, ExPEC is most commonly associated with UTIs and bloodstream infections. ST73 is a known clone within the ExPEC family (Riley, 2014). Although this ST group also includes commensal strains (Nissle 1917), very highly virulent clones, including CFT073 have been attributed to mass UTI epidemics across the globe. Previous studies have recognized this clonal lineage as the etiologic agents for both hospital and community-acquired disease. Gibreel

et al (2010) collected 300 UPEC isolates from Northwest England and used MLST methods to determine clonal lineages in efforts to identify specific clades more frequently associated with UTIs. This study found ST73 as the most common lineage frequently identified (n=43) followed by ST131 (n=37). It also identified a few ST127 clones (n=9) from both the hospital and community associated strains (Gibreel et al, 2010). Further investigation of the ST127 isolates from this study (Gibreel et al, 2010) showed particularly higher number of VF genes compared to the other study strains. In the United States, Adams-Sapper et al, (2013) analyzed UTI *E. coli* samples collected over a three-year period (2007-2010) from a hospital in San Francisco and used MLST to determine clonal lineages. Out of 249 *E. coli* (UPEC) isolates, the most common strain identified was ST131; however, ST73 and ST127 were also found in 20 and 3 of the isolates, respectively (Adams-Sapper et al, 2013). The study also utilized *fimH* typing methods to enhance differences in strains. The ST73 isolate was shown to carry alleles for *fimH*- 3, -8, -9, -60 and ST127 isolates only showed *fimH-2* (Adams-Sapper et al, 2013). More recently, Souza da-Silva et al, (2016) found ExPEC ST73 clones linked to an outbreak of community acquired urinary tract infections in both men and women in Rio de Janeiro, Brazil. This study collected 139 samples from different patients (127 women and 12 men) exhibiting UTI symptoms. After characterizing the strains using PCR-based, PFGE, and MLST methods, 21 of the isolates were found to belong to phylogenetic group B2 ST73. Additionally, these ST73 strains were also found to have higher number of virulence factors compared to the other study subtypes. (Souza da-Silva et al, 2016).

ExPEC in animals

ExPEC strains have been isolated from animals for decades and have demonstrated potential for zoonotic transfer. Luthar Beutin (1999) investigated *E. coli* acting as an extra-

intestinal pathogen in both dogs and cats. His review showed that ExPEC strains are involved in UTI infections and genital and systemic disease in dogs and cats (Beutin, 1999). Belanger et al (2010) also reviewed studies on ExPEC in human and animals to highlight how companion should be considered as reservoirs for the transmission of UPEC to humans (Belanger et al, 2010). One study aimed to investigate and characterize strains associated with UPEC in canines and potential antimicrobial resistance across the United States. LeCuyer et al, (2018) investigated 295 isolates from canine UTI samples from Washington, North Dakota, Indiana, Ohio, and California and found both ST73 and ST127 ExPEC clones (LeCuyer at al, 2018). Furthermore, this study also identified strains resistant to varied antimicrobials including ampicillin, amoxicillin, and trimethoprim-sulfamethoxazole as well as VFs in 257 of the isolates (LeCuyer et al, 2018). Liu X, et al (2015) looked to investigate major ST types in UPEC isolated from cats across the United States. This study compared 74 isolates and detected ST73 as the most frequent clone (Liu X et al, 2015). Recently, Mora et al (2017) investigated human-associated *E. coli* clonal groups from Antarctic pinnipeds and found the presence of ExPEC ST73, ST127, and ST131. This study on wild marine mammal was able to detect ExPEC lineage and identified multiple AMR phenotypes to ampicillin, cefalotin and nalidixic acid (Mora et al, 2017). This study highlighted the importance of further studying the geographical dissemination of ExPEC clones among wild marine mammals, especially in areas of low human activity.

Avian-pathogenic *E. coli (*APEC) ST73 have been isolated from chicken farms in Brazil. One study (Cunha et al, 2017), characterized clones from 27 sample strains isolated from geographically diverse poultry farm outbreaks. The study aimed to characterize the APEC strains but also investigate potential similarities to human ExPEC strains. Results showed that all the

isolates were ST73 clones and carried ExPEC VF genes in high-frequency including but not limited to: *fimH, papC, iroN, vat,* and *hlyA* (Cunha et al, 2017).

Comparative Genomics of ExPEC

Previous studies on ExPEC have aimed to characterize the phylogenetic relationships between human-associated ExPEC and avian strains (APEC). These investigations allowed for greater insight into the potential relatedness between lineages and to infer potential zoonotic risk. Moulin-Schouleur et al, (2007) used MLST and PCR-based virulence phylotyping methods to determine potential phylogenetic relatedness. By MLST, most APEC strains belonged to B2 phylogenetic group ST73, and carried VF genes associated with ExPEC strains. Statistical analyses based on prevalence of VFs among strains were done using a chi-square test and further highlighted close relationship between the human and avian strains, especially in the B2 phylogenetic group (Moulin-Schouleur et al, 2007). Johnson et al, (2008) also compared ExPEC isolates from humans (n=55), dogs (n=59), and cats (n=16) using MLST and PCR based typing methods for VF genes and their association to origin of sample. This study found ST73, ST625, and ST127 as the three major ST's found among the isolates. Furthermore, statistical analyses using multivariate ordination techniques, were used to visualize the STs along gradients highlighting a close relationship between ST73 isolates were to human strains; whereas ST127 was found to be more associated with canines (Johnson et al, 2008).

Johnson et al, (2007) authored a study that compared similarities between human and animal strains using sequencing methods. This study compared the genetic sequence of APEC (O1:K1:H7) to human ExPEC genomes to detect any similarities. To further test the hypothesis that certain APEC strains could potentially be zoonotic and cause disease in humans, specifically urinary tract infections. This study looked at 500 APEC and 500 UPEC strains and found two

major clusters: the first was primarily APEC strains containing VFs on plasmids and the second mostly carried UPEC strains with VFs chromosomally localized (Johnson et al, 2007). Furthermore, the sequenced strain (APEC O1), showed a high degree of genomic similarity to human ExPEC strains [85%] suggesting zoonotic potential. This study deposited complete sequence and annotation of APEC 01:K1:H7 in GenBank [NC008563], used for comparative analyses (see Figure 3).

Additionally, Jorgensen et al, (2019) performed a comparative genomic study between 32 avian-pathogenic *E. coli* (APEC) and 291 human ExPEC genomes to determine possible relatedness between strains. This study utilized WGS data on a large pool of ST95 (both from human and avian) isolates to compare both core and accessory genomes. Results showed that there is some genetic overlap between ExPEC and APEC clones. It also mentioned that VF associated plasmids are crucial for avian pathogenesis (colibacillosis) and were strongly associated [using Fisher exact test] with mixed APEC/human ExPEC clusters (Jorgensen et al, 2019). Rasko et al, (2008) used WGS to infer variation between commensal and pathogenic *E. coli* to better understand the genetic disparities within the core and accessory genomes. This study compared isolate sequences for different strains including (but not limited to): commensal, ETEC, EHEC, and ExPEC (human UPEC, avian APEC). Additionally, it used pangenome analyses to identify truly unique genes (TUG) within strains and found CFT073 (used as reference strain in the current study) and Ec042 to have the greatest number of TUGs (Rasko et al, 2008).

STUDY AIMS

The overall aim of this project was to use WGS to characterize and analyze nine *E. coli* isolates collected from fecal samples of the endangered SRKWs. The goal was to determine strain clonal lineage ST, subtypes, and to identify any potential antimicrobial resistance and virulence factors genes.

Aim 1: Bacterial Genomics; molecular characterization of isolates to determine strain multilocus sequence type, clonotype, and to identify antibiotic resistant genes (ARGs) and virulence factors genes (VFs).

We hypothesized that WGS of these nine isolates would yield nine high-resolution whole genomes data and allow for in silico strain identification using MLST, clonotype, and reveal antibiotic resistant genes and virulence factors.

1.1 Determine MLST profile to identify isolate strain lineage

1.2 Determine *fimH* and *fumC* subtyping (clonotype)

1.3 Determine antimicrobial resistance profile of *E. coli* ST73 and ST127

1.4 Determine virulence factor profile of *E. coli* ST73 and ST127

Aim 2: Determine genetic diversity among strains and relatedness. Determine Single Nucleotide Polymorphisms (SNPs) of core genomes to distinguish a possible clonal complex. What is the relationship between these isolates among the SRKW population? Compare core genome SNPs to reference strains to investigate relatedness.

We hypothesized that the ST73 isolate genomes will be closely related (within 20-50 SNP's) and that the ST127 genome will differ greatly. This self-relatedness and high clonal relationship support these strains having a common origin. Further comparative analyses with reference strains will highlight versatility.

2.1 Phylogenetic analysis based on core genome SNPs

2.2 Comparative genomics to other reference strains

MATERIALS AND METHODS:

Sample collection

Eleven SRKW scat samples were collected in the Salish Sea around the San Juan Islands of Washington State between August to October 2013. (**Figure 1**). The samples were tested for hormonal and toxicant measurement under the National Marine Fisheries Service Permits 532-1822-00, 532-1822, 10045, and 17344 as part of a previous study (Lundin et al, 2016). Sample collection methods were approved by the University of Washington's Institutional Animal Care and Use Committee (IACUC) under protocol 2850–08. Trained conservation canines were utilized on boats because the dog's keen olfactory system was able to pick up the scat scent from distances farther than a nautical mile (Lundin et al, 2016). The researchers followed areas downwind of where whales have been spotted, and the dog was trained to change its behavior when it picked up the fecal scent, showing subtle cues to its handler for direction. When sample was visually confirmed, a 3 to 6-foot pole with a 1-liter polypropylene beaker attached was used to scoop the sample by skimming just under it.

Bacterial screening of samples

For this study, sterile Fisher Brand cotton swabs (Fisher Scientific Waltham, MA) were inserted into the homogenized scat pellet and ~0.5 mL of the 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, vortexed for 10 sec and 0.1 mL was spread on MacConkey agar plates (Difco Laboratories, Sparks, MD) supplemented with and without antibiotics including: 25 mg/L tetracycline, 25 mg/L chloramphenicol, 25 mg/L ampicillin and incubated at 36.5 C for 24-48 h. Nine (82%) of the eleven samples tested positive for *E. coli*, and 8 (89%) of those 9 tested positive on DifcoTM Luria-Bertani media (Difco) supplemented with 25 mg/L tetracycline.

Single *E. coli* isolates from different samples and plates were verified using biochemical tests. No *E. coli* were detected on either the ampicillin or chloramphenicol supplemented media. We also attempted to isolate *E. coli* from 74 freeze-dried frozen samples stored for > 1 year without success. Additionally, the homogenized pellet samples underwent genomic profiling at NOAA NW Fisheries Center for species, sex, pod, and individual identification (Ford et al, 2011) to determine the whale of origin for each sample. Since samples were collected for a different study (Lundin et al, 2016), we were able to identify that we had isolates from whales in both the J and L pods and one unknown potential transient whale. (Table 1).

Table 1. Isolate Identification, origin of isolation, name, gender and age at collection (NOAA, and Center for Whale Research)

Isolate ID	Origin of isolation	Whale nickname[a]	Gender	Age (years)[b]
1-J28	*Orcinus orca*	Polaris	Female	23
2-J28	*Orcinus orca*	" "	" "	23
3-J8	*Orcinus orca*	Speiden	Male	80
4-UK*	*Orcinus orca*	N/A	N/A	N/A
5-L79	*Orcinus orca*	Skana	Female	34
6-J26	*Orcinus orca*	Mike	Male	40
7-J27	*Orcinus orca*	Blackberry	Male	28
8-J31	*Orcinus orca*	Tsuchi	Female	13
9-J31	*Orcinus orca*	" "	" "	13

[a] Names found at Center for Whale Research based on pod ID (Center for Whale Research)

[b]Age of whale at time of sample collection, derived from CWR

Whole Genome Sequencing

DNA extraction was done using MoBio Laboratories UltraClean® Microbial DNA Isolation Kit (Mo-Bio Laboratories, Carlsbad, CA.). The kit is designed to yield high-quality DNA from a variety of microbial isolates. Extracted DNA concentration was determined by using a Quibut (Thermofisher Technologies Inc., USA). Dual-indexed libraries were prepared using Nextera XT library prep kit (Illumina, San Diego, CA) with 1 ng of bacterial DNA and 14 amplification cycles. The kit used an engineered transposon with specific dual-indexed adapters that tagment the DNA during sequencing. The libraries were sequenced using an Illumina MiSeq. Isolates 2-J28, 3-J8, 4-UK, 6-J26, 7-J27 were sequenced on a 1 x 192 bp run, and due to logistical reasons 1-J28, 5-L79, 8-J31, and 9-J31 were re-sequenced on a 2 x 300 bp run. Raw-reads were trimmed using trimmomatic for quality, and de-novo assembled using SPAdes Genome Assembler v3.11 (Trimmomatic & SPAdes). Prokka v 1.13 was used to annotate genomes (prokka url). Assembled sequence data was deposited into NCBI GenBank under project PRNJNA338014 with accessions numbers found in (Table 2).

Table 2. Uploaded Genome Sequence Accession Links

Isolate ID	GenBank Accession #
1-J28	RQIE00000000
2-J28	RRTX00000000
3-J8	RQID00000000
4-UK*	RQIC00000000
5-L79	RQIB00000000
6-J26	RQIA00000000
7-J27	RQHZ00000000
8-J31	RQHY00000000
9-J31	RQHX00000000

MLST

MLST is the "gold standard" for *E. coli* strain identification. Isolate clonal lineages were determined using *in silico* identification based on the Achtman seven housekeeping gene scheme. MLST for *E. coli* was determined using (*adk, fumC, gyrB, icd, mdh, purA, recA*) alleles downloaded by PubMed MLST. Validation was done by uploading assembled sequences (contigs) into Center for Genomic Epidemiology's MLST finder. (https://cge.cbs.dtu.dk/services)

***fimH* Subtyping**

Clonotypes were determined using the Weismann *fumC; fimH* (type 1 fimbrial adhesin) typing method *(*Weissman et al, 2011*).* The *fimH* alleles sequences were provided by Dr. Weismann, and Geneious® 9.1.7 software (www.geneious.com) was used to perform reference alignments. For each strain assembly, only 100% identity match with allele type was chosen. The *fumC* alleles were determined from previous MLST characterization. Weissman et al, (2003) acknowledges the importance of identifying SNPs in bacterial adhesins, specifically in the *fimH* gene in *E. coli* as some variants identified in UPEC strains are associated with increased bladder colonization (Weissman et al, 2003). Validation for *fimH* allele types was done using the Center for Genomic Epidemiology *fimH* typing tools. (COMPARE project funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 643476) (https://cge.cbs.dtu.dk/services)

Antimicrobial Resistance Profile

Assembled contigs were uploaded into the *ResFinder* (https://cge.cbs.dtu.dk/services) with identity thresholds of 98% for antimicrobial resistance genes and 95% minimum template coverage length. BLAST of sequences identified were done for verification using Geneious software. Resistance phenotypes are characterized in **Appendix B.**

Virulence Factor (VF) Profile

A literature search for ExPEC VF genes was done after strain MLST typing revealed ST lineage. VF genes mentioned in reference literature were downloaded from NCBI GenBank and Geneious® 9.1.7 software was used for reference alignments. Assembled contigs were also uploaded into the VirulenceFinder phenotyping tool at 96% threshold identity (https://cge.cbs.dtu.dk/services).

Conjugation Experiments

To test whether the isolates carried mobile *tet*(B) resistance genes, conjugation experiments were done with the eight ST73 tetracycline resistant [Tc^r] isolates and with 50 mg/L rifampin 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 media supplemented with 25 mg/L tetracycline and 50 mg/L rifampin as previously described (Chopra et al, 2001).

Core-genome SNPs and comparative analyses

To determine relatedness between isolates, phylogenetic analyses were performed based on single nucleotide polymorphisms (SNPs) to detect genetic variation between strains. Core genome SNPs were identified using Snippy v 4.3.2 (https://github.com/tseemann/snippy) and known ExPEC isolate CFT073 [AE01475.1] was used as the reference strain alignment. (Welch et al, 2002). Phylogenetic analysis was based on SNP alignment and trees were constructed using Bayesian (Mr.Bayes) methods in Geneious Pro 9.1.5 under default setting. Core genome SNP comparisons were done with reference strains: APEC O1:K1:H7 [NC008563], Nissle 1917 [CP007799], and ABU83972 [CP001671]. (Johnson et al, 2007; Reister et al, 2014; Zdziarski et al 2010).

RESULTS

Whole genome sequencing (**WGS)**

Table 3 shows the characteristics of the 9 E coli genomes. Genome contig size ranged from 3,124 bp (1-J28) to 11,974 (9-J31) bp. Assembly sizes ranged from 4.8 Mb (8-J31) to 5.3 Mb (2-J28, 3-J8, 4-UK, 5-L79, 6-J26) with an average of 5.21 Mb. (Table 3).

Table 3. WGS assembly statistics for the nine *E. coli* isolates

Isolate ID	Sequencing Platform	Number of Contigs	Mean Seq. length	Min Seq. Length	Max Seq. Length	GC %	Assembly Size (Mb)
1-J28	Illumina MiSeq	1,605	3,124	128	23,736	50.9	5.2
2-J28	Illumina MiSeq	663	7,804	79	68,270	50.6	5.3
3-J8	Illumina MiSeq	542	9,517.1	95	108,554	50.6	5.3
4-UK	Illumina MiSeq	423	11,233.4	82	155,795	50.6	5.3
5-L79	Illumina MiSeq	663	7793.1	128	94,036	50.7	5.3
6-J26	Illumina MiSeq	1,060	4,860	78	59,725	50.7	5.3
7-J27	Illumina MiSeq	995	5,155.1	78	60,334	50.7	5.2
8-J31	Illumina MiSeq	1,094	4,477.3	128	38,015	51.0	4.8
9-J31	Illumina MiSeq	439	11,974	128	283,983	50.5	5.11

MLST and *fimH* typing

By *in silico* MLST, all isolates belong to ExPEC clonal lineage strain types ST73 (8/9) and ST127 (1/9). (**Table 4**). Clonotyping (CH) exhibited further divergence between clones with all ST73 isolates belonging to C24:H10 clonotype, and the ST127 isolate to C14:H2 (Table 5)

Table 4. MLST results for the nine *E. coli* isolates.

MLST								
	adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	**MLST (ST)**
1-J28	36	24	9	13	17	11	25	**73**
2-J28	36	24	9	13	17	11	25	**73**
3-J8	36	24	9	13	17	11	25	**73**
4-UK	36	24	9	13	17	11	25	**73**
5-L79	36	24	9	13	17	11	25	**73**
6-J26	36	24	9	13	17	11	25	**73**
7-J27	36	24	9	13	17	11	25	**73**
9-J31	36	24	9	13	17	11	25	**73**
8-J31	13	14	19	36	23	11	10	**127**

Table 5. Subtyping (clonotype) using *fimH* (type 1 frimbrial adhesin) and *fumC* alleles

Clonotypes			
Isolate ID	*fumC*	*fimH*	**C:H clonotype**
1-J28	24	10	**C24:H10**
2-J28	24	10	**C24:H10**
3-J8	24	10	**C24:H10**
4-UK	24	10	**C24:H10**
5-L79	24	10	**C24:H10**
6-J26	24	10	**C24:H10**
7-J27	24	10	**C24:H10**
9-J31	24	10	**C24:H10**
8-J31	14	2	**C14: H2**

AMR and VF gene profiles

ARGs identified at >98% identity include: *aadA1, sul1*, and *tet(*B) coding resistance to aminoglycoside, sulfonamide, and tetracycline, respectively for all eight ST73 isolates. No ARGs were found in the ST127 isolate in WGS data. (**Table 6**). Sixteen VFs genes were identified at >96% identity include: adhesins (*iha, papC, sfaS),* toxins (*sat, vat, pic, hlyA, cnf1*), siderophores *(iroN, ireA, iutA, fyuA*), serum survival/protectins (*iss, ompT*), capsule (*kpsM*) and pathogenicity island marker (*malX).* (**Table 7**). Additionally, a part of the gene for *catA1* coding resistance to chloramphenicol was detected in all ST73 isolates. Only ~480 bp of 660 bp was found (76% of the template length); however, these strains did not express phenotypic resistance and this gene was not included in results.

Table 6. Antimicrobial Resistance Profile

Antimicrobial Resistance Profile		
Isolate ID	AR Genes[a]	Predicted Phenotype
1-J28	*aadA*1, *sul*1, *tet*(B)	Aminoglycoside, Sulfonamides, Tetracycline
2-J28	*aadA*1, *sul*1, *tet*(B)	Aminoglycoside, Sulfonamides, Tetracycline
3-J8	*aadA*1, *sul*1, *tet*(B)	Aminoglycoside, Sulfonamides, Tetracycline
4-UK*	*aadA*1, *sul*1, *tet*(B)	Aminoglycoside, Sulfonamides, Tetracycline
5-L79	*aadA*1, *sul*1, *tet*(B)	Aminoglycoside, Sulfonamides, Tetracycline
6-J26	*aadA*1, *sul*1, *tet*(B)	Aminoglycoside, Sulfonamides, Tetracycline
7-J27	*aadA*1, *sul*1, *tet*(B)	Aminoglycoside, Sulfonamides, Tetracycline
9-J31	*aadA*1, *sul*1, *tet*(B)	Aminoglycoside, Sulfonamides, Tetracycline
8-J31	none	none

[a] *aadA*1 and *sul*1 found by WGS; *tet*(B) found by WGS, PCR and some were a few of the *tet*(B**)** genes were mobilized by conjugation.

Table 7. Virulence Profile

Virulence Factor Genes					
Isolate ID	Adhesives	Toxins	Siderophores	Serum survival	Misc.
1-J28	*iha, papC, sfaS*	*sat, hlyA*	*iutA, fyuA*	*iss, omp*T	*kpsM, malX*
2-J28	*iha, papC, sfaS*	*sat, vat, pic*	*iroN, ireA, iutA, fyuA*	*iss, ompT*	*kpsM, malX*
3-J8	*iha, papC, sfaS*	*sat, vat, pic*	*iroN, ireA, iutA, fyuA*	*iss*	*kpsM, malX*
4-UK*	*iha, papC, sfaS*	*sat, vat, pic*	*iroN, ireA, iutA, fyuA*	*iss*	*kpsM, malX*
5-L79	*iha, papC, sfaS*	*sat, pic*	*iroN, ireA, iutA, fyuA*		*kpsM, malX*
6-J26	*iha, papC, sfaS*	*sat, pic*	*iroN, ireA, iutA, fyuA*	*iss*	*kpsM, malX*
7-J27	*iha, papC, sfaS*	*vat, pic, hlyA*	*iutA, ireA, fyuA*		*kpsM, malX*
9-J31	*iha*	*sat, vat, pic*	*iroN, ireA, iutA, fyuA*	*iss*	*kpsM, malX*
8-J31	*sfaS*	*cnf1, vat*	*iroN, ireA, fyuA*	*iss, ompT*	*kpsM*

Conjugation Experiment

Three isolates (1-J28, 4-UK, 5-L79) were shown to transfer the *tet*(B) to recipient *E. coli* HB101 at a frequency of 5.9 X 10^{-5}, and 9.0 X 10^{-8} respectively (**Table 9**). Transconjugants were verified by growth on LB media supplemented with 25 µg/mL tetracycline and 50 µg/mL rifampin. This suggests a plasmid location though no plasmids were identified using plasmid methods for isolation.

Table 9. Conjugation Experiments

Isolate ID	Resistant Phenotype	Antibiotic Gene	Transfer frequency into HB101[a]	Tet gene in transconjugants
1-J28	Tetracycline	*tet*(B)	5.9×10^{-5}	*tet*(B)
2-J28	Tetracycline	*tet*(B)	$< 1 \times 10^{-9}$	none
3-J8	Tetracycline	*tet*(B)	$< 1 \times 10^{-9}$	none
4-UK*	Tetracycline	*tet*(B)	9.0×10^{-8}	none
5-L79	Tetracycline	*tet*(B)	1.1×10^{-8}	*tet*(B)
6-J26	Tetracycline	*tet*(B)	$< 1 \times 10^{-9}$	none
7-J27	Tetracycline	*tet*(B)	$< 1 \times 10^{-9}$	none
9-J31	Tetracycline	*tet*(B)	$< 1 \times 10^{-9}$	*tet*(B)

[a] $< 1 \times 10^{-9}$ = no transconjugants were detected

SNP comparison

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 [>3,000 SNP differences], while ST127 was distinct with > 26,000 SNP differences (**Figure 2**). Comparative core genome SNP analyses with ABU83972 strains and ST73 isolates also showed relatedness [~2,540 SNP differences] (**Figure 3**). Analysis of Nissle 1917 and study ST73 isolates showed less relation [~3,098 - 3,123 SNP differences] (**Figure 4**). The biggest difference in SNP analyses was seen between with the APEC O1:K1:H7 strain reference [24,651 – 26,219 SNP differences]. (Figure 5).

DISCUSSION

In this study, WGS was used to identify and characterize ExPEC clonal strains ST73 and ST127 isolated from wild marine mammals, specifically the endangered Southern Resident killer whales. This is the first study to analyze SRKW *E. coli* using next-generation sequencing technology. Previously, a respiratory microbiome study on SRKW used traditional biochemical and PCR-based methods (Raverty et al, 2017). The current study identified ARGs and VF genes, while disk diffusion and MIC tests were performed for phenotypic verification of resistance. The ST73 isolates were phenotypically resistant to tetracycline and contained *tet*(B) gene [by PCR and WGS]. These eight ST73 isolates were also resistant to aminoglycosides (spectinomycin and streptomycin), and sulfonamides (sulfisozaxole) and carried corresponding ARGs [**Appendix A**]. Many of the VFs identified in this study were consistent with VF genes frequently associated with ExPEC mobile elements [*ompT, iutA, iroN* and *iss* genes] (Nicholson et al, 2016). The distribution of ARGs found in the study isolates were also similar [*tet gene*] to those identified in the metagenomic profile of the Puget Sound (Port et al, 2012), and also to the resistance profiles [tetracycline] identified from the respiratory microbiota of the SRKW (Raverty et al, 2017).

ExPEC clonal groups [ST73, ST127, and ST131] have also been isolated in faecal samples collected from wild Antarctic marine mammals (pinnipeds) (Mora et al, 2017). The ExPEC isolates from the Antarctic study were resistant to ampicillin, cefalotin, and nalidixic acid, which differed from the current study suggesting that geographical locations differ in AMR dissemination. Both studies identified carriage of similar VF genes including: *fimH, iroN, cnf1, sfaA,* and *hlyA* (Mora et al, 2017).

SNP analyses of core genomes of the study isolates showed high degree of genetic relatedness among the eight whale *E. coli* ST73 isolates (Figure 2). This highlights that the

clones were able to spread to six different individual whales and within at least two different pods. Comparative core-genome SNP analyses between study ST73 and ST127 isolates to known strains [CFT073, ABU83972 ST73, Nissle 1917 ST73, APEC O1 (bird isolate)] showed that ST73 were more closely related [fewer SNP differences] to CFT073, and ABU83972 isolates [**Figure 2, 3**]. CFT073 (*E. coli* O6:H1:K2) is a highly virulent human strain, first isolated from a patient with pyelonephritis at the University of Maryland, and its sequenced genome showed over sixty unique segments encoding VF genes (Welch et al, 2002). In contrast, ABU83972, an asymptomatic bacteriuria isolate cultured from a schoolgirl, is often used as a therapeutic agent for bladder colonization. Although its sequenced genome shows close relation to both CFT073 and Nissle 1917, the difference in the loss of VF genes in its genome is hypothesized to be important for strain survival (Zdziarski et al 2010). The ST73 and ST127 from the study showed lower relatedness to the Nissle 1917 and least with APEC isolates **(figure 4, 5**). Nissle 1917, a probiotic bacterium identified during the first World War by an army doctor and sequenced in 2014, does not carry VF genes either and is known to show antagonistic activity against other pathogenic gut bacteria *in vivo* (Reister et al, 2014). Henker et al, (2007) showed that using this probiotic strain to colonize children suffering from diarrhea resulted in reduced disease. The APEC isolate came from a bird suffering from colibacillosis and was the least related to the orca *E. coli* [>24,000 SNPs].

The biggest limitation of the study is the low number of *E. coli* isolates characterized. In addition, of the 85 samples cultured, 74 of these were freeze-dried frozen and no *E. coli* could be isolated. Furthermore, sequenced reads from Illumina are not good at finding plasmids, so it is possible that some plasmid genes were not identified.

CONCLUSION

This study helps to advance our understanding of the spread of AMR in microbes (*E. coli)* and marine mammals in the Salish Sea, and demonstrates the need for increased microbial surveillance efforts with the declining SRKW population. It stresses the importance of fostering antimicrobial stewardship in the Pacific Northwest by working to prevent dissemination of human-associated AMR microbes to this critically at-risk population. Previous studies on the ST73 and ST127 strains of E coli have found associations with disease in humans and companion animals; however, without proper veterinary assessments, or urine samples, it was not possible to determine whether the whales were sick at the time of fecal collection.

Within the previous decade, many studies have investigated potential factors affecting the health of the Salish Sea orcas. The Southern Resident killer whales are currently experiencing significant health stressors including high levels of toxic chemicals [PCBs], reduced nutritional supply, and disturbance from human pressures [vessel and noise] (Lundin et al, 2016, Wasser et al, 2017). One of the biggest stressors, lack of prey availability, directly affects the population's recovery by reducing fecundity. Wasser et al, (2017) genotyped fecal samples to determine pregnancy success and failure based on hormonal measures. This study found that the SRKW had a 69% pregnancy failure rate during time of investigation (Wasser et al, 2017). This past summer [2018], one of the SRKW from the J pod (J35; Scarlet) gave birth to a calf that died within the hour, and she proceeded to carry the body on top of her head for over two weeks in mourning and grief, drawing international attention (Center for Whale Research, 2019). This was the first calf to be born alive in three years to this pod, and having the offspring pass away confirms everyone's fear: the SRKWs are headed for extinction.

This study was guided by the One Health principle that we all share the responsibility of making sure our planet is healthy for all beings (not just human) in future generations. The SRKW are still in decline and face continued threats to their habitat and their health. In March 2018, Washington governor Jay Inslee announced an unprecedented investment to save these whales, including a recovery agenda and over $1 billion of funding support (Puget Sound partnership, 2018). The Southern Resident Orca Task force will collaborate with federal, tribal, state and local partners to outline recommendations for the recovery of these whales including cleanup of their environment and reduction of underwater noise from boat and ships; however, only time will tell if any of these measures are successful. The findings from this study will serve as evidence to support further investigations on microbial prevalence of the SRKW;

FIGURES

Figure 1. Location of where samples were taken in The San Juan Islands. Geographic representation of sample collection area. Red dots indicate were faecal samples were collected in the Salish Sea [created using Adobe Illustrator]

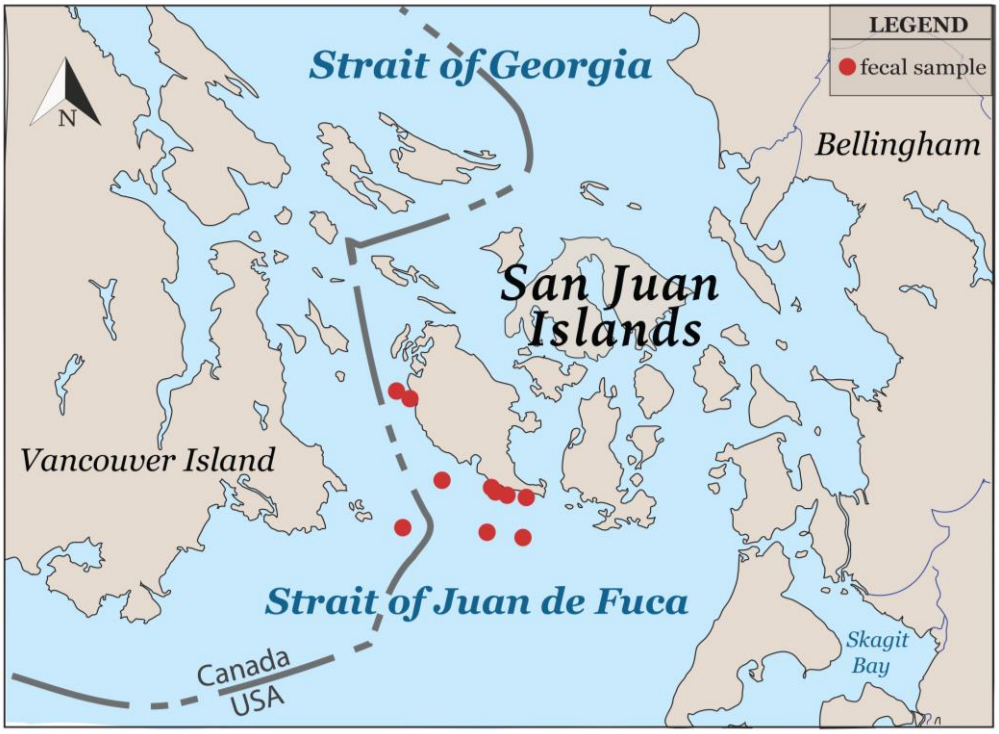
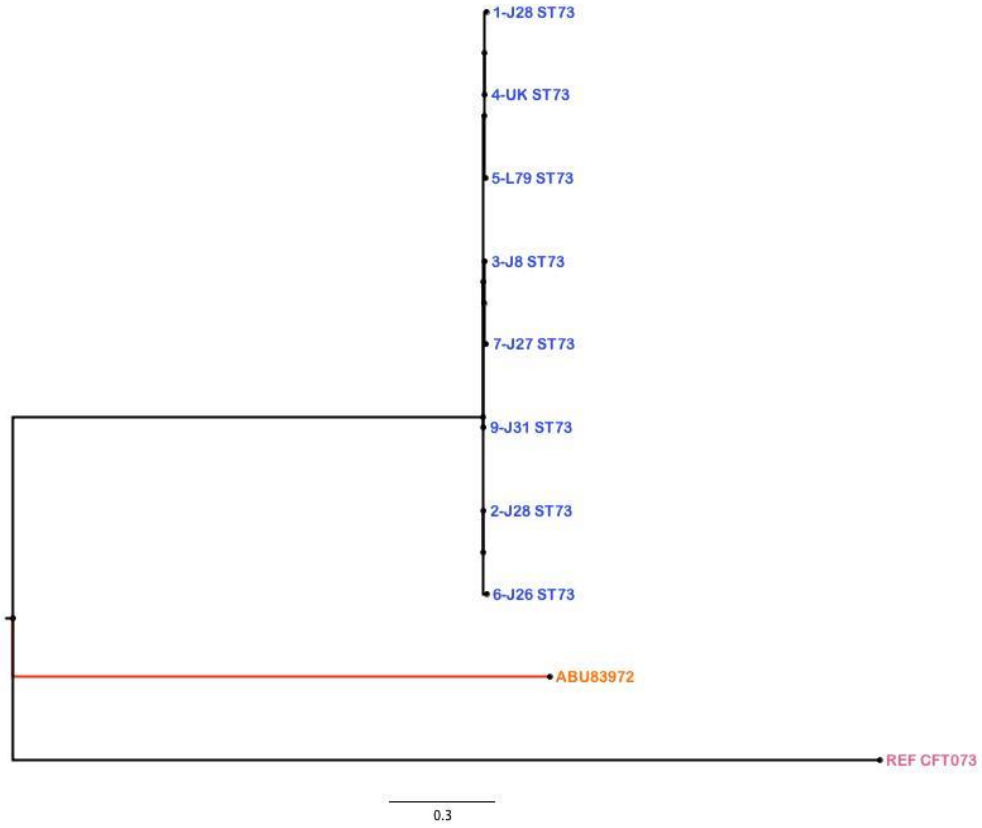


Figure 2. Core genome SNP phylogeny between ST73 and ST127 strains (CFT073 as reference)

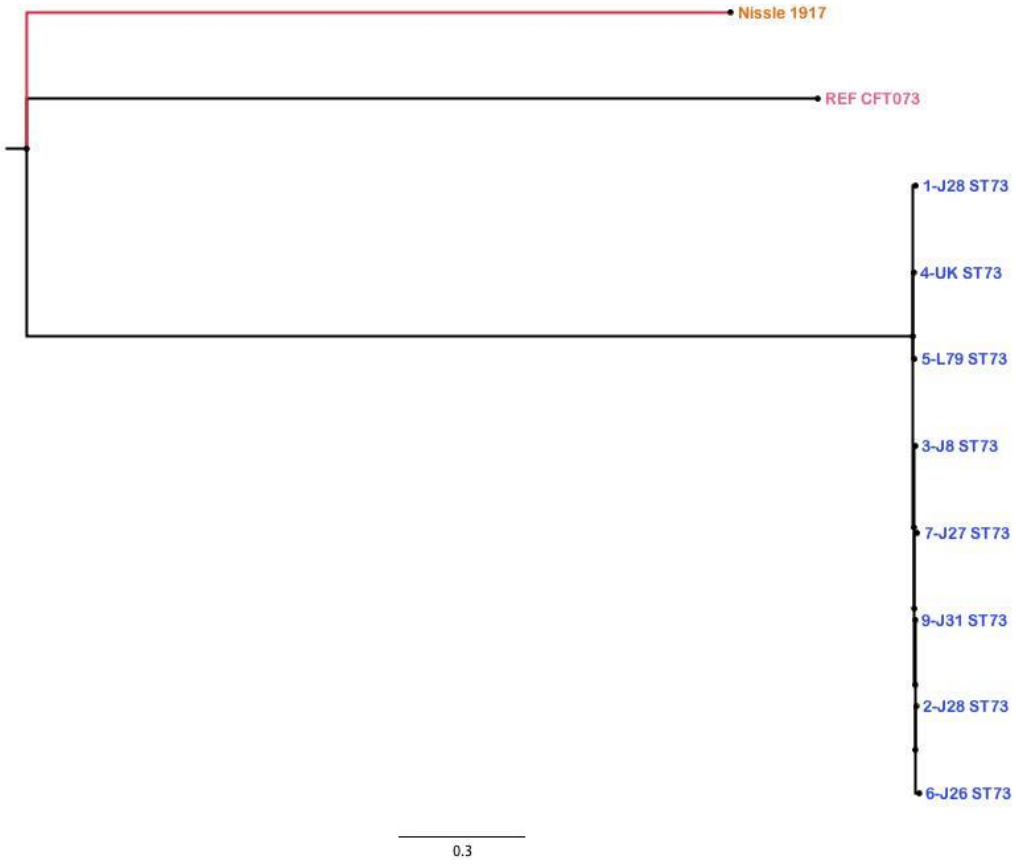
	1-J28	2-J28	3-J8	4-UK	5-L79	6-J26	7-J27	9-J31	8-J31
1-J28									
2-J28	42								
3-J8	49	21							
4-UK	31	19	26						
5-L79	46	30	43	23					
6-J26	68	40	53	47	60				
7-J27	57	31	42	40	46	57			
9-J31	36	8	21	17	28	34	23		
8-J127	26,187	26,177	26,186	26,176	26,191	26,205	26,194	26,173	
CFT073	3,076	3,062	3,067	3,057	3,076	3,088	3,079	3,058	25,667

Figure 3. Core genome SNP comparison between ST73 isolates and ST127 with ABU83972



	1-J28	2-J28	3-J8	4-UK	5-L79	6-J26	7-J27	9-J31
1-J28								
2-J28	38							
3-J8	45	17						
4-UK	27	15	24					
5-L79	44	28	39	21				
6-J26	67	37	48	44	59			
7-J27	56	28	37	37	46	57		
9-J31	35	5	16	14	27	34	23	
ABU83972	2,813	2,520	2,529	2,519	2,536	2,549	2,540	2,519

Figure 4. Core genome SNP comparison with Nissle 1917

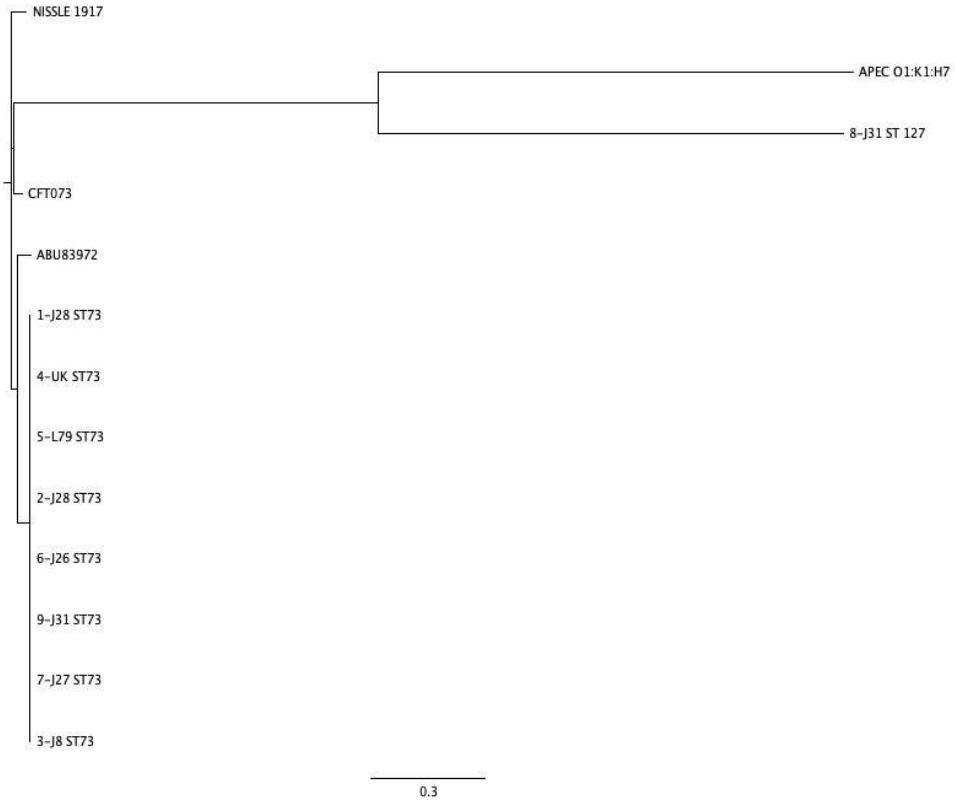


	1-J28	2-J28	3-J8	4-UK	5-L79	6-J26	7-J27	9-J31
1-J28								
2-J28	38							
3-J8	44	18						
4-UK	26	14	24					
5-L79	42	30	40	18				
6-J26	65	37	47	41	59			
7-J27	55	29	37	33	46	57		
9-J31	34	6	16	10	27	45	23	
Nissle 1917	3,116	3,098	3,106	3,096	3,112	3,123	3,115	3,094

Figure 5. Core genome SNP comparison with APEC 01:H1:K7

	1-J28	2-J28	3-J8	4-UK	5-L79	6-J26	7-J27	9-J31
1-J28								
2-J28	39							
3-J8	46	19						
4-UK	29	18	25					
5-L79	44	29	42	23				
6-J26	66	35	48	44	59			
7-J27	55	26	37	36	46	56		
9-J31	36	5	18	15	27	43	21	
APEC	24,582	24,563	24,572	24,561	24,576	24,588	24,577	24,558

Figure 6. Core genome SNPs combined



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APPENDICES

A. **Antimicrobial Susceptibility test MIC**

Kirby Baur Disk Diffusion method (Difco Laboratories, Division Becton-Dickinson) was performed according to Clinical and Laboratory Standards Institute (CLSI) protocol with E. coli ATCC 25922 as a control (CLSI). Isolates were streaked on Mueller-Hinton (MH) agar forming a lawn, and the following antibiotic disks were aseptically placed. Different groups of antibiotics used: chloramphenicol (25 µg), cefepime (30 µg), spectinomycin (50 µg), ciprofloxacin (5 µg), gentamicin (10 µg), tetracycline (30 µg), and trimethoprim-sulfamethoxazole (30µg) (Becton-Dickinson Microbiology Systems, Franklin Lakes, NJ). Sample plates were incubated for 18-24 h at 36.5° C. Results were recorded according to CLSI standards.

A.

	Amikacin (30ug)		Ampicillin (10ug)		Cefepime (30ug)		Cefotaxime (30ug)		Ceftazidime (30ug)		Chloramphenicol (30ug)		Gentamicin (10ug)		Imipenem (10ug)		Meropenem (10ug)		Tetracycline (30ug)		Ciprofloxacin (5ug)	
E. coli ID	S I R	≥ 17 mm 15-16 mm ≤ 14 mm	I R	≥ 17 mm 14-16 mm ≤ 13 mm	S I R	≥ 18 mm 15-17 mm ≤ 14 mm	S I R	≥ 26 mm 23-25 mm ≤ 22 mm	S I R	≥ 21 mm 18-20 mm ≤ 17 mm	S I R	≥ 18 mm 13-17 mm ≤ 12 mm	S I R	≥ 15 mm 13-14 mm ≤ 12 mm	S I R	≥ 23 mm 20-22 mm ≤ 19 mm	S I R	≥ 23 mm 20-22 mm ≤ 19 mm	S I R	≥ 15 mm 12-14 mm ≤ 11 mm	S I R	≥ 21 mm 16-20 mm ≤ 15 mm
ATCC 25922	23mm	Susceptible	19mm	Susceptible	31mm	Susceptible	30mm	Susceptible	28mm	Susceptible	22mm	Susceptible	19mm	Susceptible	27mm	Susceptible	31mm	Susceptible	23mm	Susceptible	35 mm	Susceptible
1-J28	25mm	Susceptible	21mm	Susceptible	35mm	Susceptible	35mm	Susceptible	30mm	Susceptible	26mm	Susceptible	24mm	Susceptible	31mm	Susceptible	35mm	Susceptible	6mm	Resistant	35mm	Susceptible
2-J28	23mm	Susceptible	21mm	Susceptible	33mm	Susceptible	33mm	Susceptible	30mm	Susceptible	25mm	Susceptible	22mm	Susceptible	28mm	Susceptible	35mm	Susceptible	6mm	Resistant	35mm	Susceptible
3-J8	22mm	Susceptible	20mm	Susceptible	34mm	Susceptible	31mm	Susceptible	30mm	Susceptible	24mm	Susceptible	21mm	Susceptible	27mm	Susceptible	30mm	Susceptible	6mm	Resistant	36mm	Susceptible
4-UK	22mm	Susceptible	20mm	Susceptible	33mm	Susceptible	33mm	Susceptible	29mm	Susceptible	24mm	Susceptible	20mm	Susceptible	29mm	Susceptible	33mm	Susceptible	6mm	Resistant	36mm	Susceptible
5-L79	22mm	Susceptible	22mm	Susceptible	33mm	Susceptible	31mm	Susceptible	28mm	Susceptible	23mm	Susceptible	21mm	Susceptible	28mm	Susceptible	31mm	Susceptible	6mm	Resistant	38mm	Susceptible
6-J26	22mm	Susceptible	20mm	Susceptible	34mm	Susceptible	32mm	Susceptible	30mm	Susceptible	21mm	Susceptible	20mm	Susceptible	27mm	Susceptible	29mm	Susceptible	6mm	Resistant	36mm	Susceptible
7-J27	22mm	Susceptible	18mm	Susceptible	32mm	Susceptible	31mm	Susceptible	30mm	Susceptible	22mm	Susceptible	19mm	Susceptible	28mm	Susceptible	30mm	Susceptible	6mm	Resistant	35mm	Susceptible
8-J31	20mm	Susceptible	21mm	Susceptible	31mm	Susceptible	31mm	Susceptible	28mm	Susceptible	24mm	Susceptible	19mm	Susceptible	27mm	Susceptible	29mm	Susceptible	23mm	Susceptible	35mm	Susceptible
8-731	22mm	Susceptible	21mm	Susceptible	31mm	Susceptible	29mm	Susceptible	27mm	Susceptible	21mm	Susceptible	21mm	Susceptible	28mm	Susceptible	30mm	Susceptible	6mm	Resistant	35mm	Susceptible

B. AMR genes and accession numbers

ID	Resistance Gene	Identity	Phenotype	Accession no.
1-J28	*aadA1*	99.75%	Aminoglycoside resistance	JQ414041
1-J28	*sul1*	100%	Sulphonamide resistance	CP002151
1-J28	*tet*(B)	100%	Tetracycline resistance	AF326777
2-J28	*aadA1*	99.75%	Aminoglycoside resistance	JQ414041
2-J28	*sul1*	100%	Sulphonamide resistance	AY224185
2-J28	*tet*(B)	100%	Tetracycline resistance	AP326777
3-J8	*aadA1*	99.75%	Aminoglycoside resistance	JQ414041
3-J8	*sul1*	100%	Sulphonamide resistance	AY224185
3-J8	*tet*(B)	100%	Tetracycline resistance	AF326777
4-UK	*aadA1*	99.75%	Aminoglycoside resistance	JQ414041
4-UK	*sul1*	100%	Sulphonamide resistance	CP002151
4-UK	*tet*(B)	100%	Tetracycline resistance	AF326777
5-L79	*aadA1*	99.48%	Aminoglycoside resistance	JQ41041
5-L79	*sul1*	100%	Sulphonamide resistance	CP002151
5-L79	*tet*(B)	100%	Tetracycline resistance	AF326777
6-J26	*aadA1*	99.74%	Aminoglycoside resistance	JQ414041
6-J26	*sul1*	100%	Sulphonamide resistance	CP002151
6-J26	*tet*(B)	100%	Tetracycline resistance	AF326777
7-J27	*aadA1*	99.75%	Aminoglycoside resistance	JQ414041
7-J27	*sul1*	100%	Sulphonamide resistance	CP002151
7-J27	*tet*(B)	100%	Tetracycline resistance	AF326777
9-J31	*aadA1*	99.75%	Aminoglycoside resistance	JQ414041
9-J31	*sul1*	100%	Sulphonamide resistance	CP002151
9-J31	*tet*(B)	100%	Tetracycline resistance	AF326777

C. Virulence Genes & Accession Numbers

Virulence Gene	Accession Number
iss	AE014075
iha	CP001671
sat	CP001671
vat	AE014075
ireA	CP000468
pic	AE014075
iroN	AE01475
sfaS	CP000243
*ompT**	P09169
*hlyA**	Q8G972
*iutA**	P14542
*fyuA**	P0C2M9
*kpsM**	P23889
*malX**	P19642
*cnf1**	Q46962
*papC**	P07110

*sequences were found in UniProt (https://www.uniprot.org/uniprot)