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# One Health Approach for Identification of Sources/Reservoir of Multidrug Resistant Bacteria in Wild Animals and their Environment

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PRELIMINARY COMMUNICATION

Bacteria such as extra-intestinal pathogenic E. coli (ExPEC) and methicillin-resistant S. aureus (MRSA) are important opportunistic pathogens. They might belong to pandemic, epidemic and/or sporadic clones. Some of the clones are associated with humans, others are associated with wild and/or domestic animals. Some clones are shared by both and may be found contaminating the environment. In these studies, we examined the spread of ExPEC from feces of Southern Resident Killer Whale (SRKW; Orcinus orca) that are associated with human diseases. We also examine MRSA isolates in wild rhesus macaques (Macaca mulatta), their environment and from humans. This One Health Approach aims to better understand the sources/reservoirs and possible transmissions of potential pathogens between animals, humans and their shared environment.

**Keywords:** Antibiotic resistance genes, extra-intestinal pathogenic E. coli, killer whales, MLST, MRSA, rhesus macaques

## 1. Introduction

Antibiotic resistant bacteria are identified in wild animals from birds to insects with increasing frequency [1]. The occurrence of these bacteria is often associated with human influence on the environment, spreading from people to animals and the environment or on occasion from animals or the environment back to people. The sharing of these antibiotic resistant bacteria has been documented primarily in land animals and birds, with limited work on marine mammals or primates [1]. In the current studies, we first examine the presence of E. coli from Southern Resident Killer Whales (SRKW), which are apex predators. This cetacean serves as a sentinel for its environment, providing valuable indices of the overall health of the Salish Sea (Puget Sound) boundary waters shared by Washington State, USA and British Columbia, Canada [4, 5]. The second study characterizes methicillin resistant Staphylococcus aureus (MRSA) isolated from wild rhesus macaques (Macaca mulatta) living in and around temple areas of the Kathmandu valley in Nepal, where human-macaque interaction is common, along with the shared environment in the valley and a few clinical strains from a local hospital.

## 2. Material and Methods

### *2.1. E. coli and DNA.*

*E. coli* was isolated from fresh fecal samples collected from the endangered SRKW population (*O. orca*) in the Salish Sea [Puget Sound] in 2013. The red dots in Figure 1 indicate sampling sites off the San Juan Islands. 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 deployed on boats to detect specific SRKW fecal 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 [4]. Sterile Fisher Brand cotton swabs (Fisher Scientific Waltham, MA) were inserted into the homogenized faecal pellet and

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**Figure 1:** Location of whale samples. Red dots show where fecal samples were collected.

~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, vortexed for 10 seconds 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, or 25 mg/L ampicillin and incubated at 36.5 oC for 24-48 h. Nine of the eleven samples tested positive for E. coli, and 8 of the 9 grew on DifcoTM Luria-Bertani media (Difco) supplemented with 25 mg/L tetracycline [5].

E. coli isolates from different samples and plates were identified using standard biochemical tests. No E. coli were detected on either the ampicillin or chloramphenicol supplemented media. No E. coli could be isolated from 74 freeze-dried frozen faecal samples stored for >1 year. 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 Qubit (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. 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 [5].

*2.2. Isolation of MRSA and characterization.*

A total of 227 saliva samples from wild rhesus macaques (Macaca mulatta) living in and around temple areas of the Kathmandu valley were collected during Feb 2018. The areas; Bajrayogini, Nilbarahi, Pashupatinath, Swyambhu and Thapathali were sampled with Bajrayogini being the most distant site from the city of Kathmandu. The collection technique involved an adaptation of the non-invasive oral sampling method in Evans [2] and Roberts [10] using SalivaBio Children's Swabs (Salimetrics LLC, State College PA, USA). Swabs were soaked in a sterile glucose solution (10% w/v) and thrown to the macaques, which they then chewed and discarded for immediate collection. Macaque saliva samples (n=13) and environmental samples (n=19) isolated near temple areas in Kathmandu were included in the study. Environmental surface samples (n=218) were collected in July 2018 from Bajrayogini, Nilbarahi, Pashupatinath, Swyambhu and Thapathali. High touch surfaces were selected at the temple sites. Solid surfaces were swabbed with sponges to collect the bacteria on the surfaces as previously described [6]. Five random isolates from wound infected patients were obtained by some of the Nepali authors for comparison with the primate and environmental MRSA. Beside their methicillin resistance, nothing was previously known about these isolates. Ethical approval was also obtained from the Kist Medical College and Teaching Hospital, Imadol, Lalitpur, Nepal, for the clinical MRSA isolates. All 37 isolates were characterized using The Alere StaphyType® DNA microarrays [7, 8]. The Abbott StaphyType® DNA microarray based assay was used for all isolates as previously described [7, 8]. The microarray typing includes 334 target sequences and ~170 separate genes and allelic variants including species markers, SCCmec, capsule, agr group typing markers, common antibiotic resistance genes, toxins and microbial surface components recognizing adhesive matrix molecules [MSCRAMM] genes [8].

## 3. Results

Whale E. coli. Nine distinct isolates were recovered and analyzed from seven SRKW individuals with whole genome sequencing, de novo assembly. Eight samples had multidrug resistant ExPEC ST73 clonotype C24:H10 isolates taken from 7 individuals from 3 pods (Table 1). The ninth isolate was not antibiotic resistant and was ExPEC ST127 clonotype C12:H2. All isolates carried a variety of virulence genes which differed between the ST73 isolates and between the ST73 and the ST127 isolates (Table 2). Previous studies showed that the Puget Sound (Salish Sea), home to the SRKW, is contaminated with multiple ARGs and antibiotic residues, especially near waste water treatment plant discharge sites [9]. The SRKW food source, Chinook salmon, also carry antibiotic residues in their tissue.

Nepalese MRSA. From the 227 primate saliva samples, 13 (5.7%) were MRSA positive. Multiple positive primate samples were identified in four of the five areas sampled: Bajrayogini [n=4], Pashupatinath [n=3], Swyambhu [n=2] and Thapathali [n=4]. In contrast, from the 218

**Table 1:** LST, Clonotype, Antibiotic Resistance of Whale E. coli. 4-UK* this may be a transient whale.

| Isolate ID | ST | Clonotype | Resistance Genes | Predicted Phenotypes |
|---|---|---|---|---|
| 1-J28 | ST73 | C24:H10 | aadA1,sul1, tet(B) | Aminoglycoside, Sulfonamides, Tetracycline |
| 2-J28 | ST73 | C24:H10 | aadA1,sul1, tet(B) | Aminoglycoside, Sulfonamides, Tetracycline |
| 3-J8 | ST73 | C24:H10 | aadA1,sul1, tet(B) | Aminoglycoside, Sulfonamides, Tetracycline |
| 4-UK* | ST73 | C24:H10 | aadA1,sul1, tet(B) | Aminoglycoside, Sulfonamides, Tetracycline |
| 5-L79 | ST73 | C24:H10 | aadA1,sul1, tet(B) | Aminoglycoside, Sulfonamides, Tetracycline |
| 6-J26 | ST73 | C24:H10 | aadA1,sul1, tet(B) | Aminoglycoside, Sulfonamides, Tetracycline |
| 7-J27 | ST73 | C24:H10 | aadA1,sul1, tet(B) | Aminoglycoside, Sulfonamides, Tetracycline |
| 9-J31 | ST73 | C24:H10 | aadA1,sul1, tet(B) | Aminoglycoside, Sulfonamides, Tetracycline |
| 8-J31 | ST127 | C12:H2 | N/A | N/A |

**Table 2:** Virulence factors Whale E. coli

| Isolate ID | Adhesins | Toxins | Siderophores | Serum survival | Misc. |
|---|---|---|---|---|---|
| 1-J28 | iha, papC, sfaS | sat, ,hlyA | iutA, fyuA | iss | 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 |

environmental samples, 19 (8.7%) were MRSA positive cultured from all five areas sampled; Bajrayogini [n=3], Nilbarahi [n=2], Pashupati [n=6], Swyambhu [n=5] and Thapathali [n=3]. All 37 MRSA isolates were further characterized.

Twenty-three (62%) MRSA were CC22 SCCmec type IVa previously found in Nepalese macaque of human origin and isolated from monkey (n=4; 31%), environmental (n=14; 74%), and human (n=5; 100%) samples [10]. Eight monkey MRSA were CC361 SCCmec type IVa. One MRSA isolated from a monkey and environment were CC88 SCCmec type V, previously found in Nepalese swine samples [10]. The remaining environmental MRSA included one each, CC121 SCCmec type V, and CC772 SCCmec type V, all of human origin and two CC779 SCCmec type V, potentially a novel clone. All 37 MRSA carried the bla gene, 31 carried the aacA -aadD, 25 dfrA and 21 erm(C) genes. All CC22 isolates carried the aacA-aadD, dfrA and 17 carried the erm(C) genes, while 2 MRSA from macaque, 3 MRSA from environmental and 1 human MRSA lacked the erm(C) gene. The one macaque and environmental CC88 MRSA both carried the aacA-aphD gene but only the macaque MRSA carried the aphA3 and sat resistance genes, neither previously identified in primate MRSA [10] (Table 3). Among the 23 CC22 MRSA, 21 carried the PVL locus and tst virulence gene which is unusual and include all the monkey and human isolates and 12 of 14 environmental isolates [3]. This current study suggests that humans are the source of the MRSA identified in both the macaques and the environment and may be linked to humans feeding the primates and/or the primates living in close proximity to the humans.

## 4. Conclusions

As human populations continue to expand, so do opportunities for transmission of pathogens between humans and wildlife. We documented such transmission in a marine mammal and Old World terrestrial primate.

The study on antibiotic resistant E. coli isolated from SRKW helps to advance our understanding of the spread of AMR E. coli in the Salish Sea. It also demonstrates the need for increased microbial surveillance efforts of the declining SRKW population. Previous studies on the ST73 and ST127 have been associated with disease in humans and companion animals; however, without proper veterinary assessments, or urine samples it was not possible to determine if the whales were sick at the time of fecal collection. Therefore it is unknown if carriage of ExPEC isolates increases the risk of disease in the SRKW and/or if they contribute to the ongoing decline of this endangered species. This is the first time it was determined that Orca whales can carry antibiotic resistant potentially pathogenic strains of E. coli. The ExPEC isolates in the SRKW most

**Table 1:** MRSA strains, SCCmec types and resistance genes[a]. [a] The table shows only genes which were found at least once in this study. Genes which were not present in any of the study strains are: mecC, blaZ SCCmec XI, erm(A), erm(B), lnu(A), mef(A), vat(A), vat(B), vga (A), vga(A), vgb(A), far1, fusC, mupR, tet(K), tet(M), cat, cfr, fexA, qacA, qacC , vanA, vanB and vanZ.
[b] Subtypes are referred to by designations of reference strains that yield identical SCC patterns on the arrays used. GenBank references for these reference strains are as follows: MW2, BA000033.2; IS-105, AHLR; Bengal Bay (CMFT1723), HF569096.1; GR1, AJLX.

| Strain | Host | N | mecA | SCCmec subtype[b] (number subtyped) | blaZ | erm(C) | msr(A) | mph(B) | aacA-aphD | aadD | aphA3 | sat | dfrA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CC22-MRSA-IV (PVL+/ tst1+) | Rhesus | 4 | 4 | IVa as in MW2 (1) | 4 | 2 | - | - | 4 | - | - | - | 4 |
| | Environment | 12 | 12 | IVa as in MW2 (1) | 12 | 9 | - | - | 12 | - | - | - | 12 |
| | Human | 5 | 5 | IVa as in MW2 (2) | 5 | 4 | - | - | 5 | - | - | - | 5 |
| CC22-MRSA-IV (tst1+) | Environment | 1 | 1 | IVa as in MW2 (1) | 1 | - | - | - | 1 | - | - | - | 1 |
| CC22-MRSA-IV (PVL) | Environment | 1 | 1 | IVc as in IS-105 (1) | 1 | - | - | - | 1 | 1 | - | - | 1 |
| CC88-MRSA-V | Rhesus | 1 | 1 | V as in Bengal Bay (1) | 1 | 1 | 1 | 1 | 1 | - | 1 | 1 | - |
| CC88-MRSA-V (PVL+) | Environment | 1 | 1 | V as in Bengal Bay (1) | 1 | 1 | - | - | 1 | - | - | - | - |
| CC121-MRSA-VT | Environment | 1 | 1 | VT as in GR1 (1) | 1 | 1 | - | - | 1 | - | - | - | - |
| CC361-MRSA-IV | Rhesus | 8 | 8 | IVa as in MW2 (1) | 8 | - | - | - | - | - | - | - | - |
| CC772-MRSA-V (PVL+) | Environment | 1 | 1 | V as in Bengal Bay (1) | 1 | - | 1 | 1 | 1 | - | 1 | 1 | - |
| CC779-MRSA-VT | Environment | 2 | 2 | VT as in GR1 (1) | 2 | 2 | - | - | 2 | - | - | - | - |

likely are either directly acquired from pollution in the Salish Sea, and/or from their salmon diet. How well these isolates survive in the marine environment is also not known since in general E. coli are not salt tolerant.

The current study on MRSA isolated from Nepal suggests that humans are the source of the MRSA identified both in the macaques and the environment and may be linked to humans feeding the primates. The Neplaese MRSA strains (CC22, CC361, CC772) indicate epidemiological links to other countries within the Indian subcontinent and to the Middle East. Strains that have been detected in monkeys have been found in humans, either in this or in other studies. For the environmental CC779-MRSA-VT, no conclusions can be drawn due to a lack of data. However, this lineage has been found in humans before (see above) and we are not aware of any published observations on its presence in animals. In conclusion, it can be speculated that the detection of MRSA in Nepalese Rhesus can be attributed at least in a majority of cases to contamination/infection during contacts with humans or to human offal. Thus, humans can not only be infected with zoonotic pathogens by close contact with wild animals; they also might transmit human pathogens into wildlife, posing a possible hazard to wild animals whose population are already endangered and under stress. However, the impact on the monkeys might be limited in this particular case as a related species of macaques seemed to be rather resistant towards PVL [3].

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## Submission declaration

Part of the ExPEC work has been previously published [10] but the current technical report provides new information that was not published in the short note and is used to contrast with MRSA work that has recently been published [11].

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