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1 **Human-associated Extended Spectrum β -Lactamase**
2 **(ESBL) in the Antarctic**

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Abstract

Escherichia coli with extended spectrum β -lactamase (ESBL) type CTX-M resistance were isolated from water samples collected close to research stations in Antarctica. The isolates had *bla*_{CTX-M-1} and *bla*_{CTX-M-15} genotypes and sequence types (ST) indicative of a human-associated origin. This is the first record of ESBL-producing enterobacteria from Antarctica.

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35 *Enterobacteriaceae* with extended spectrum β -lactamase (ESBL) enzymes is
36 increasing in clinical and veterinary medicine, posing a threat to future healthcare (6). ESBL-
37 producing bacteria are now commonly isolated both in industrialized and in developing
38 countries (6, 12), and dissemination into the environment has been observed (1). The most
39 common ESBLs are TEM, SHV and CTX-M – each with a different evolutionary origin (4,
40 9). These classes are subdivided into gene-groups and to date >300 different ESBL variants
41 have been described (8). In recent years the CTX-M has become the most widespread class
42 (5). The Antarctic continent is the last comparative pristine ecosystem with a small human
43 population restricted to research bases, primarily located on the Antarctic Peninsula. Human
44 activities are regulated by the Antarctic Treaty to reduce interference with the unique wildlife
45 and the impact of human-associated microorganisms should be minimal (2). However,
46 contrary to this intention, human-associated pathogens have been indentified in Antarctic
47 wildlife (18). Here we report the presence of human associated ESBL-producing *Escherichia*
48 *coli* with bla_{CTX-M} genes in water sampled close to research bases on the Antarctic Peninsula
49 and the South Shetlands Islands, providing the first cases of ESBL in the region.

50 The fieldwork was conducted during the 2011 austral summer (January-
51 February) when the bases Bernardo O'Higgins on the Antarctic Peninsula, the Fildes Bay
52 (King George Island) and the Arturo Prat (Greenwich Island) in the South Shetlands Islands
53 were visited for sampling. Water samples were collected from the sea surface in concentric
54 circles from the stations' sewage outlets (10, 25, 50, 100, 200 and 300 m). Each of the 123
55 water samples (125 ml volume) was filtered through sterile 0.45 μ m membrane filters which
56 were cultured on chromogenic selective plates (ChromoCult, Merck, Darmstadt, Germany)
57 for detection of *E. coli* and coliform flora. The filters were then placed in Luria broth
58 (phosphate-buffered saline including 0.45% Na-citrate, 0.1% MgSO₄, 1% (NH₄)₂SO₄ and
59 4.4% glycerol) and stored at -70°C. Additionally, we sampled a total of 400 fresh faecal

60 droppings from gentoo penguins (*Pygoscelis papua*) from colonies close to the bases. All
61 samples were shipped on dry-ice to Sweden for further analyses.

62 In order to isolate *E. coli* for characterization, all water and penguin samples
63 were cultivated on Uriselect4 plates (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and
64 colonies were identified by conventional biochemical tests. Randomly chosen 71 *E. coli*
65 isolates (40 from water and 31 from penguin samples) to determine antibiotic susceptibility
66 profiles using the EUCAST disc diffusion method in order to receive an overall picture of
67 resistance in the material (15). The panel included 11 antibiotic discs: tetracycline 30 µg/disc,
68 ampicillin 10 µg/disc, streptomycin 10 µg/disc, chloramphenicol 30 µg/disc, nalidixic acid 30
69 µg/disc, cefadroxil 30 µg/disc, fosfomicin 50 µg/disc, tigecycline 15 µg/disc,
70 trimethoprim/sulphamethoxazole 1.25/23.75 µg/disc, nitrofurantoin 100 µg/disc and
71 mecillinam 10 µg/disc (all antibiotics from Oxoid Ltd, Cambridge, UK). The *E. coli* strain
72 ATCC25922 was used as control.

73 Only one *E. coli* isolate from a penguin (Bernardo O'Higgins base) had a
74 resistant phenotype, in this case to chloramphenicol. In the water samples, three isolates were
75 resistant to at least one antibiotic compound and several were resistant to two or more
76 antibiotics (Table 1). The most frequently observed resistance was to ampicillin, found in
77 eleven isolates, followed by tetracycline (six isolates), streptomycin (four isolates), and
78 trimethoprim/sulphamethosaxole (four isolates). One *E. coli* was resistant to nalidixic acid
79 (Table 1). The presence of ESBL-producing bacteria was investigated by enriching all
80 samples in Brain Heart Infusion broth (Becton Dickinson, Franklin Lakes, USA)
81 supplemented with vancomycin (16 mg/L, ICN Biomedicals Inc. Aurora, USA) for 18h at
82 37°C, and subsequently inoculate on chromID™ ESBL plates (bioMérieux, Marcy L'Etoile,
83 France). Ten *E. coli* ESBL isolates were retrieved and ESBL production was confirmed in
84 each isolate with a cefpodoxime/cefpodoxime + clavulanic acid double disk test (MAST

85 Diagnostics, Bootle, UK). These isolates were all positive for the CTX-M ESBL but negative
86 for TEM and SHV in specific qPCR (13, 14). The PCR products were sequenced on both
87 strands using the following primers: CTX-F (5'TCCCAGAATAAGGAATCCCAT-3') and
88 CTX-R1 (5'CCCATTCCGTTTCCGCTA-3'). The resulting consensus sequences were
89 compared with published sequences in the NCBI database. Four *E. coli* isolates, all from
90 Fildes Bay, carried the *bla*_{CTX-M-1} gene, while sex isolates (four from Arturo Prat and two
91 from Bernardo O'Higgins) carried the *bla*_{CTX-M-15} gene. The ESBL isolates were further
92 analysed by multi locus sequencing typing (MLST) using a modified version of previously
93 described procedures (19). Sequence types (STs) and putative associated sources were
94 retrieved from an on-line MLST *E. coli* database (<http://mlst.ucc.ie/mlst/dbs/Ecoli/>). Six
95 different STs (ST131, ST227, ST401, ST410, ST685 and ST937) two to four at each research
96 station were identified (Table 2).

97 The *bla*_{CTX-M-1} and *bla*_{CTX-M-15} genes are both human-associated ESBL genotypes
98 (16, 19). Genotyping by MLST corroborated the association with human gastrointestinal
99 biota, as the majority of the STs belonged to widespread disease-associated genotypes,
100 including *E. coli* ST131 carrying *bla*_{CTX-M-15} gene, a worldwide disseminated human clinical
101 clone (11). These findings constitute the first records of ESBL-producing bacteria in
102 Antarctica, and given that they were isolated from all the sampled bases it seems that human-
103 associated bacteria are discharged into the environment more than occasionally. The
104 emergence of CTX-M has been referred to as one of the most striking examples of rapid,
105 global dissemination of plasmid-mediated resistance determinants among bacterial pathogens
106 (16). Nosocomial and community spread of ESBL genotypes are well described in humans (3)
107 but there are less studies on the presence of these genes in bacteria in the environment (1).
108 The efforts to suppress further development and dispersal of resistance are focused on
109 reducing the consumption of antibiotics. However, the factors that maintain, and perhaps

110 facilitate further resistance dispersal in natural environments (e. g. sewage treatment) are
111 given less attention. The mechanisms behind the success of CTX-M are still to be determined
112 (16). Therefore, the finding of CTX-M-producing bacteria in Antarctic seawater and the
113 finding of high prevalence of CTX-M in certain wild bird populations needs to be considered
114 carefully. In addition to ESBL-producing isolates, several non-ESBL *E. coli* with resistance to
115 common antibiotics were found in the water samples, including isolates with resistance to up
116 to 4 compounds (Table 1). An important question to ask is whether the human-associated
117 bacteria found close to human activities infect Antarctic wildlife. None of the sampled
118 penguins were positive for ESBL-producing bacteria, and only one *E. coli* exhibited antibiotic
119 resistance, indicating that at the time of sampling human-associated enterobacteriaceae was
120 not common in the sampled penguins. The risk of a bird to be colonized by *E. coli* has been
121 shown to vary with the proximity to environments influenced by human activity (10). An
122 earlier study in Antarctica indicated a 17 % prevalence of *E. coli* in penguins (17). The lower
123 prevalence of *E. coli* (~8%) in our samples could indicate infrequent interactions between
124 penguin and human microbiota, especially since the penguin strains were highly susceptible
125 to antibiotics and showed no presence of ESBL. However, penguins are but one part of the
126 ecosystem, and compared to more opportunistic feeders, such as kelp gulls (*Larus*
127 *dominicanus*), skuas (*Stercorarius* spp.) and snowy sheatbills (*Chionis albus*), they may be
128 less exposed to human-associated bacteria. An opportunistic diet has previously been
129 associated with the presence of human-associated enteropathogens in birds (7). At present, we
130 cannot tell whether ESBL-producing bacteria are present in Antarctic wildlife, or the
131 consequences that would have for animal health. However, the presence of anthropogenic
132 bacteria in the Antarctic environment is worrisome in itself and indicative of how widespread
133 the global antibiotic resistance situation has become. The existing precautions and sewage
134 treatment at the research bases seem inadequate. Clearly, increased sampling in the Antarctic

135 biome is warranted, as well as increased efforts in reducing potential leakage of bacteria from
136 human activities.

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205
 206 **Table 1:** The number of resistant *E. coli* isolates from seawater samples collected at three
 207 research bases in the Antarctic region, 2011.

Antibiotic compound	Number of <i>E. coli</i> isolates per location		
	Greenwich Island	Antarctic Peninsula	King George Island
Nalidixic Acid	0	0	1
Streptomycin	1	1	2
Tetracycline	5	0	1
Ampicillin	7	2	2
Trimethoprim/Sulphametoaxsole	1	1	2
Susceptible to all compounds	0	8	20
Resistant to 1 compound	1	1	1
Resistant to 2 compounds	5	0	0
Resistant to 3 compounds	1	1	1
Resistant to 4 compounds	0	0	1
Total	7	10	23

208
 209 **Table 2:** Genotypic and phenotypic characteristics of ESBL-producing *E. coli* isolates from
 210 water samples collected at three research bases in the Antarctic region, 2011.

Location	ESBL genotype			MLST		Phenotypic Resistance
	CTX-M	TEM	SHV	ST	Clonal Complex	
Greenwich Island	-15	-	-	ST410	ST23 complex	Na ¹ , Te ² , Amp ³ , Cfr ⁴
	-15	-	-	ST410	ST23 complex	Na, Te, Amp, Cfr
	-15	-	-	ST410	ST23 complex	Na, Te, Amp, Cfr
	-15	-	-	ST685	Unassigned	Na, Amp, Cfr
Antarctic Peninsula	-15	-	-	ST937	Unassigned	Na, Te, Amp, Cfr
	-15	-	-	ST131	Unassigned	Na, Te, Amp, Cfr
King Georges Island	-1	-	-	ST401	Unassigned	Amp, Cfr
	-1	-	-	ST131	Unassigned	Amp, Cfr
	-1	-	-	ST410	ST23 complex	Amp, Cfr
	-1	-	-	ST227	ST10 complex	Amp, Cfr

211
 212 ¹Nalidixic Acid (Na), ²Tetracyclin (Te), ³Ampicilin (Amp), ⁴Cefadroxil (Cfr).