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SHORT NOTE

Isolation of drug-resistant *Salmonella enterica* serovar enteritidis strains in gentoo penguins from Antarctica

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Abstract Anthropogenic activity in Antarctica constitutes a continuous risk for the introduction of infectious diseases into indigenous wildlife populations. Penguin colonies living close to human settlements or inhabiting in areas considered for tourism could be facing a greater threat of infection. Fecal samples from *Pygoscelis* penguins (*Pygoscelis* spp.) were collected from different sites within Antarctic Peninsula and South Shetlands Islands in order to assess the presence of *Salmonella enterica*. Bacterial identification and characterization was performed applying biochemical and molecular techniques. Isolates were tested for antimicrobial resistance by the disk diffusion method, and PCR analyses were used for detection of resistance and virulence-associated genes. Four samples (1.74%) from *P. papua* were found to be positive to *S. enterica* serovar Enteritidis strains. All of them showed phenotypic antimicrobial resistance to at least three antimicrobials, and shared a similar gene profile through PCR. Results in this study urgently call for improvements in sanitary standards for waste disposal and sewage treatment in Antarctica. To our knowledge, this is the first study to report antimicrobial resistance in *S. enterica* isolated from Antarctic wild species.

Keywords *Salmonella enterica* · Antimicrobial resistance · Antarctica · Gentoo penguins

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Introduction

Extreme environmental conditions and relative geographic isolation resulted in low pathogenic pressure for Antarctic wildlife during their evolutionary history, which make them especially vulnerable to introduction of infectious diseases (Grimaldi et al. 2015). Several studies have investigated the presence of pathogenic organisms in Antarctic penguins and discussed possible implications for anthropogenic activity (Iveson et al. 2009; Grimaldi et al. 2015). In fact, the growing human influence in Antarctica represents a threat for wildlife populations, as inappropriate waste disposal, tourism, and commercial ships, may contribute to disseminate exotic microorganisms in the Antarctic communities (Iveson et al. 2009). On the other hand, migratory birds have also been addressed as a potential source of infection for Antarctic wildlife, as they travel back and forth to different continents and islands with the risk of encountering polluted areas during their path (Vigo et al. 2011). *Salmonella enterica* is a frequent cause of gastrointestinal infections for both humans and animals, with the latter acting as healthy carriers or becoming ill (Vigo et al. 2011). In Antarctica and sub-Antarctic islands, different *S. enterica* serotypes with implications for human and wildlife health have been isolated from Gentoo (*Pygoscelis papua*) and Adelie penguins

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(*P. adeliae*) (Iveson et al. 2009; Vigo et al. 2011; Grimaldi et al. 2015). Along with avian cholera (*Pasteurella multocida*), *S. enterica* has been recognized as an important menace to penguin populations in Antarctica (Grimaldi et al. 2015).

Resistance to antimicrobials is a relevant factor for bacterial diseases in wildlife, as it is a valuable indicator of human-related contamination (Miller et al. 2009). In Antarctica, antibiotic resistance has been scarcely detected (De Souza et al. 2006; Bonnedahl et al. 2008; Miller et al. 2009), with *Escherichia coli* as the sole bacteria isolated directly from Gentoo penguins to display some degree of resistance to an antibiotic (Hernández et al. 2012). However, *E. coli* detected in water samples from the environment showed low and intermediate resistance to multiple antibiotics, with the potential risk of contaminating wildlife (Rabbia et al. 2016). *Salmonella* has not yet found to be resistant to any antimicrobial in Antarctica. Still, serotypes Enteritidis and Agona have been recently reported to be resistant to tetracycline and ceftiofur, respectively, in Magellanic penguin populations (*Spheniscus magellanicus*) distributed at high latitudes in South America (Dougnac et al. 2015). Therefore, the aim of this work was to investigate presence of *Salmonella* serotypes in Antarctic penguins and evaluate their susceptibility to different antibiotics.

Materials and methods

Study site and sample collection

During summer (January–February) in years 2014 and 2016, a total of 230 fresh feces samples were obtained

Table 1 Samples collected from Antarctic penguins and analyzed for *Salmonella enterica* isolation

Penguin species	Collection site	Year	N° samples	Coordinates
Pygoscelis papua	Base Bernardo O'Higgins*	2014	50	63°19′S, 57°51′W
	Kopaitic island	2014	50	63°17′S, 57°55′W
	Base Gabriel González Videla	2014	50	64°49′S, 62°51′W
	Narebski point	2016	14	62°12′S, 58°45′W
	Fort point	2016	14	62°28′S, 59°48′W
	Ardley island	2016	4	62°12′S, 58°55′W
P. antarctica	Fort point	2016	14	62°28′S, 59°48′W
	Ardley island	2016	14	62°12′S, 58°55′W
P. adeliae	Ardley island	2016	20	62°12′S, 58°55′W

* Positives samples to *Salmonella enterica* were collected from this location

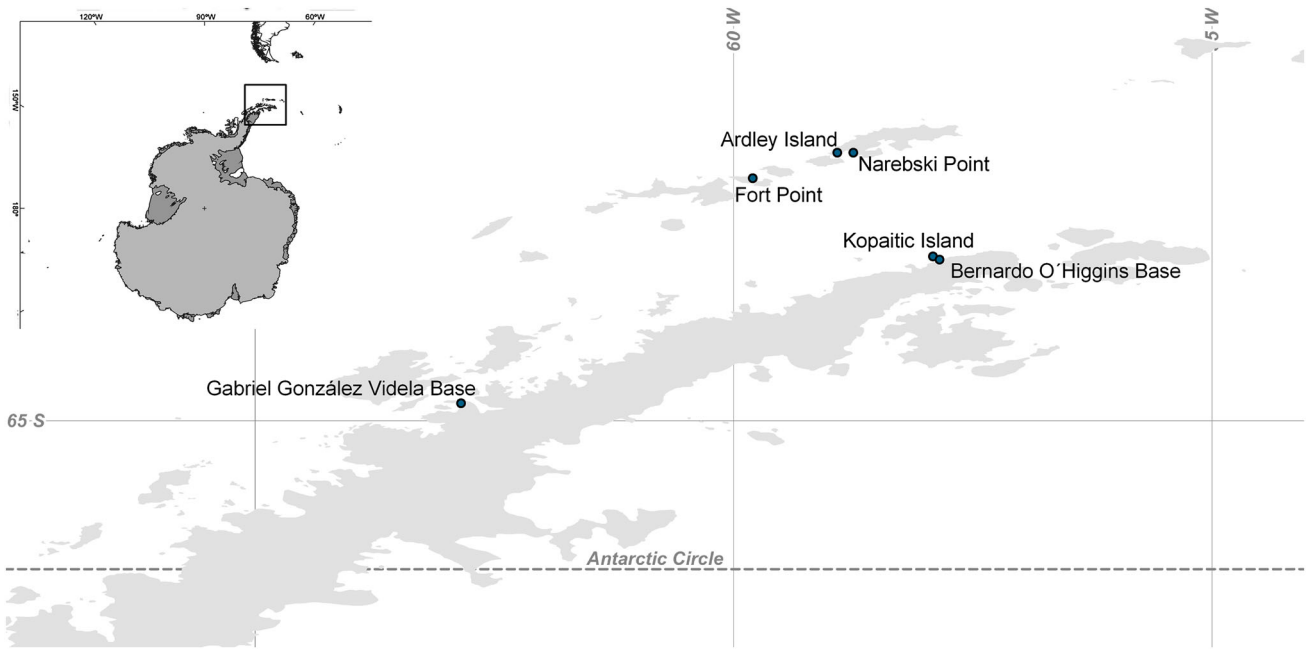

Fig. 1 Map with locations where samples were collected

through cloacal swabbing from penguins distributed along Antarctic Peninsula and South Shetland Islands (Table 1; Fig. 1). Capture and handling of birds was performed following indications in CCAMLR Ecosystem Monitoring Program (CCAMLR 2004). Penguins were released close to their colonies after sampling. All samples were kept in Cary Blair medium and stored at 4 °C for further analysis in Laboratory of Infectious Diseases, Universidad de Chile.

Bacteria isolation, serotyping, antimicrobial resistance analysis

Bacteria isolation was performed following protocols described in Fresno et al. (2013). Biochemical tests and *invA* gene detection by PCR were performed in order to identify suspicious colonies (Malorny et al. 2003). *S. enterica* strains were serotyped in the Chilean Institute of Public Health according to Kauffmann–White scheme (Grimont and Weill 2007). In addition, samples were tested for antimicrobial susceptibility by the disk diffusion method following CLSI criteria (CLSI 2008, 2011). This test was applied using the following antimicrobials (μg/disk): amoxicillin–clavulanic acid (20/10), ceftriaxone (30), cefotaxime (30), gentamicin (10) trimethoprim–sulfamethoxazole (1.25/23.75) tetracycline (30), ciprofloxacin (5), cefradine (30), ceftiofur (30), sulfisoxazole (300), chloramphenicol (30), and enrofloxacin (10). *E. coli* ATCC25922 was used as control strain.

Table 2 PCR primers used for detection of antimicrobial resistance and virulence-associated genes

Target	Function*	Primer	Sequence (5′–3′)	Product (bp)	References
tet(A)	AR	D	gctacatcctgcttgccttc	210	Ng et al. (1999)
		R	catagatcgccgtgaagagg		
tet(B)	AR	D	ttggttaggggcaagttttg	659	Ng et al. (1999)
		R	gtaatgggccaataacaccg		
tet(G)	AR	D	gctcggtggtatctctgctc	468	Ng et al. 1999
		R	agcaacagaatcgggaacac		
bla_{PSE-1}	AR	D	tttggttccgcgctatctg	131	Carlson et al. (1999)
		R	tactccgagcaccaaatccg		
bla_{CMY}	AR	D	gacagcctctctttctccaca	1000	Randall et al. (2004)
		R	tggaacgaaggctacgta		
aadB	AR	D	gagcgaaatctgccgctctgg	319	Randall et al. (2004)
		R	ctgttacaacggactggccgc		
aacC	AR	D	ggcgcgatcaacgaatttatccga	489	Lynne et al. (2008)
		R	ccattcgatgccgaaggaaacgat		
intA	AR	D	ggcatccaagcagcaagc	1200	Randall et al. (2004)
		R	aagcagacttgacctgat		
pefA	V	D	cctgtgacctgaccacttctg	418	Huehn et al. (2010)
		R	gtaagccactgcgaaagatg		
spvC	V	D	ctccttgcacaaccaaatgcg	570	Huehn et al. (2010)
		R	tgtctctgcatttcaccaccatc		
sirA	V	D	tgcgcctggtgacaaaactg	313	Huehn et al. (2010)
		R	actgacttcccaggctacagca		
gipA	V	D	acgactgagcaggctgag	518	Huehn et al. (2010)
		R	ttggaaatggtgacggtagac		
SEN1417	V	D	gatcgctggctggtc	670	Pan et al. (2009)
		R	ctgaccgtaatggcga		
prot6e	V	D	gcctaaggttagtgtgactctc	579	Huehn et al. (2010)
		R	ctagcagccgttggtatcc		
pagK	V	D	accatcttcactatattctgctc	151	Huehn et al. (2009)
		R	acctctacacattttaaaccaatc		
STY3676	V	D	accttgagcacatcaagtct	347	Litrup et al. (2010)
		R	aaacaggttttcagggttct		

* *AR* antimicrobial resistance; *V* Virulence

PCR analyses

Detection of genes associated to antimicrobial resistance and virulence was performed using primers described in Table 2. DNA extraction was performed by the High Pure PCR Template preparation Kit® (Roche, Mannheim, Germany) following the manufacturer's recommendations. All PCR reactions contained 2.5×10^{-2} U of Platinum Taq DNA polymerase® per µL; 1x Taq buffer; 1.5 mM $MgCl_2$ (Invitrogen, Sao Paulo, Brasil); dinucleotides at concentrations of 200 µM each; and each primer at a concentration of 0.5 µM. PCR reactions included a first denaturation step at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. After agarose gel electrophoresis, amplified DNA segments were stained with GelRed® (Biotium, Hayward, CA) and detected using an ultraviolet (UV) transilluminator. *Salmonella* Reference Collection B (SARB) 16, 17, 18, 19, and SAL4630 strains (Toro et al. 2016) were used as positive controls.

Results and discussion

From 230 fecal samples analyzed, four (1.74%) were positive to *S. enterica* serovar Enteritidis. Analysis performed in samples from 2015 to 2016 did not account for *Salmonella* detection. These results show an overall infection rate of 2.04% in Gentoo penguins (4/196), as it was found only in Base Bernardo O'Higgins in 2014. This detection rate is similar to results from other works (0.38–3%) involving penguin species in the sub-Antarctic Islands and Antarctica (Iveson et al. 2009; Vigo et al. 2011; Dougnac et al. 2015). Whether this infection represents a natural transmission cycle for *Salmonella*, or an exogenous contamination source for penguin populations, are questions that remain for future studies. However, all isolates corresponded to multidrug-resistant (MDR) bacteria (Table 3), i.e., resistant to three or more antimicrobial classes, most of which are classified by WHO as critically important antimicrobials for human medicine (WHO 2013). This result probably evidences the influence of external variables (Bonnedahl et al. 2008), such as was previously determined in penguins inhabiting near human settlements in Antarctica (Miller et al. 2009). In this work, resistance against tetracycline was observed in the four isolates, a high incidence that has also been obtained with other *S. enterica* serovar Enteritidis strains detected in penguins (Dougnac et al. 2015) and in seabirds along the Chilean coast (Fresno et al. 2013; Retamal et al. 2015). On one hand, migratory birds could facilitate the introduction and spreading of such kind of pathogens into native communities elsewhere (Iveson et al. 2009). On the other hand, resistance against ceftiofur, which is for veterinary use only, and resistance against ciprofloxacin, which is recommended for invasive *Salmonella* infections in humans (WHO 2013), strongly suggest the presence of an anthropogenic effect in Antarctica, as all positive samples to *S. enterica* were collected from Gentoo penguin colonies close to the Base Bernardo O'Higgins. Furthermore, all isolates belonged to serotype Enteritidis, which is the most frequent in Chilean and South American human populations (Hendriksen et al. 2011). Besides, antimicrobial-

Table 3 Antimicrobial resistance in *S. enterica* serovar Enteritidis isolates

Antimicrobial	Isolate ID			
	122	123	124	125
Amoxicillin–clavulanic acid	R	–	–	–
Ceftriaxone	R	–	–	R
Cefotaxime	R	–	–	–
Ceftiofur	–	R	R	R
Gentamicin	R	R	–	R
Ciprofloxacin	R	–	–	–
Enrofloxacin	R	–	–	R
Sulfisoxazole	–	–	R	R
Chloramphenicol	R	–	R	R
Tetracycline	R	R	R	R

R Resistant. – Susceptible

Table 4 PCR detection of resistance and virulence-associated genes

Target gene	Function*	Isolate ID			
		122	123	124	125
tetA	AR	–	–	–	–
tetB	AR	–	–	–	–
tetG	AR	+	+	+	+
Bla_{PSE}	AR	–	–	–	–
Bla_{CMY}	AR	–	–	–	–
aadB	AR	+	+	+	+
aacC	AR	–	–	–	–
intA	AR	+	+	+	+
pefA	V	+	+	+	+
spvC	V	+	+	+	+
sirA	V	–	–	–	–
gipA	V	–	–	–	–
SEN1417	V	+	+	+	+
prot6e	V	+	+	+	+
pagK	V	+	+	+	+
STY3676	V	–	–	–	–

* *AR* antimicrobial resistance; *V* Virulence; + detection; – no detection

resistant *E. coli* had been previously isolated from both Gentoo penguins and seawater associated to the same human settlement, including two *E. coli* genotypes commonly related with human gastrointestinal biota (Hernández et al. 2012). Thus, this indicates that human sewage might be a constant sanitary risk for wildlife.

Our PCR analyses detected specific determinants of resistance to tetracycline (*tetG*) and gentamicin (*aadB*) (Table 4), with a good (although not perfect) correlation to phenotypic results (Table 3). In addition, the presence of a class I integron in all isolates might explain their MDR phenotypes (Randall et al. 2004), representing a rapid mechanisms for MDR spreading among other *Salmonella* or pathogenic bacteria circulating in this environment. Other genetic traits should be evaluated to elucidate the observed phenotypic diversity of resistance, since our PCR analyses showed a complete identity among isolates (Table 4). In trying to determine genotypic relatedness, we look for virulence-associated genes which previously had been variably detected among *Salmonella* strains (Pan et al. 2009; Huehn et al. 2010). Again, no differences were observed between isolates (Table 4). Although more discriminative techniques should be tested, our PCR analyses are suggesting a common source of *S. enterica* serovar Enteritidis in the sampled penguin population around the Base Bernardo O'Higgins.

Here, we document antimicrobial resistance in *S. enterica* isolates for the first time in Antarctica, with all of them displaying MDR phenotypes. This is extremely alarming for its significance to wildlife conservation and public health in such continent. Although Gentoo penguins seem to be asymptomatic carriers of *Salmonella*, environmental variations due to climate change or human activities could provide stressful conditions for animals, therefore increasing the risk of developing disease.

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References

Bonnedahl J, Olsen B, Waldenström J, Broman T, Jalava J, Huovinen P, Österblad M (2008) Antibiotic susceptibility of faecal bacteria in Antarctic penguins. Polar Biol 31:759–763. doi:10.1007/s00300-008-0430-3

Carlson SA, Bolton LF, Briggs CE, Hurd HS, Sharma VK, Fedorka-Cray PJ, Jones BD (1999) Detection of multiresistant *Salmonella typhimurium* DT104 using multiplex and fluorogenic PCR. Mol Cell Probes 13:213–222

CCAMLR (2004) CCAMLR Ecosystem monitoring program: Standard methods. Commission for the Conservation of Antarctic marine living resources. https://www.ccamlr.org/en/science/ccamlr-ecosystem-monitoring-program-cemp. Accessed 28 May 2016

CLSI (2008) Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard, 3rd Ed. CLSI document M31-A3. CLSI, Wayne, Pennsylvania

CLSI (2011) Performance standards for antimicrobial susceptibility Testing; twenty-first informational supplement. Clinical and Laboratory Standards Institute. http://vchmedical.ajums.ac.ir/_vchmedical/documents/CLSI%202011.pdf. Accessed 20 Jan 2017

De Souza MJ, Nair S, Bharathi PL, Chandramohan D (2006) Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic marine waters. Ecotoxicology 15:379–384. doi:10.1007/s10646-006-0068-2

Dougnac C, Pardo C, Meza K, Arredondo C, Blank O, Abalos P, Vidal R, Fernández A, Fredes F, Retamal P (2015) Detection of *Salmonella enterica* in Magellanic penguins (*Spheniscus magellanicus*) of Chilean Patagonia: evidences of inter-species transmission. Epidemiol Infect 143:1187–1193. doi:10.1017/S0950268814002052

Fresno M, Barrera B, Gornall V, Lillo P, Paredes N, Abalos P, Fernández A, Retamal P (2013) Identification of diverse *Salmonella* serotypes, virulotypes, and antimicrobial resistance phenotypes in waterfowl from Chile. Vector Borne Zoonotic Dis 13:1–4. doi:10.1089/vbz.2013.1408

Grimaldi WW, Seddon PJ, Lyver POB, Nakagawa S, Tompkins DM (2015) Infectious diseases of Antarctic penguins: current status and future threats. Polar Biol 38:591–606. doi:10.1007/s00300-014-1632-5

Grimont PA, Weill FX (2007) Antigenic formulae of the Salmonella serovars, 9th edn. WHO collaborating centre for reference and research on Salmonella, Paris

Hendriksen RS, Vieira AR, Karlsmose S, Lo Fo Wong DM, Jensen AB, Wegener HC, Aarestrup FM (2011) Global monitoring of Salmonella serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: results of quality assured laboratories from 2001 to 2007. Foodborne Pathog Dis 8:887–900. doi:10.1089/fpd.2010.0787

Hernández J, Stedt J, Bonnedahl J, Molin Y, Drobni M, Calisto-Ulloa N, Gomez-Fuentes C, Astorga-España MS, González-Acuña D, Waldenström J, Blomqvist M, Olsen B (2012) Human-associated extended-spectrum β-lactamase in the Antarctic. Appl Environ Microbiol 78:2056–2058. doi:10.1128/AEM.07320-11

Huehn S, Bunge C, Junker E, Helmuth R, Malorny B (2009) Poultry-Associated *Salmonella enterica* subsp. enterica Serovar 4,12:d:- reveals high clonality and a distinct pathogenicity gene repertoire. Appl Environ Microbiol 75:1011–1020. doi:10.1128/AEM.02187-08

Huehn S, La Ragione RM, Anjum M, Saunders M, Woodward MJ, Bunge C, Helmuth R, Hauser E, Guerra B, Beutlich J, Brisabois A, Peters T, Svensson L, Madajczak G, Litrup E, Imre A, Herrera-Leon S, Mevius D, Newell DG, Malorny B (2010) Virulotyping and antimicrobial resistance typing of Salmonella enterica serovars relevant to human health in Europe. Foodborne Pathog Dis 7:523–553. doi:10.1089/fpd.2009.0447

Iveson JB, Shellam GR, Bradshaw SD, Smith DW, Mackenzie JS, Mofflin RG (2009) *Salmonella* infections in Antarctic fauna and island populations of wildlife exposed to human activities in coastal areas of Australia. Epidemiol Infect 137:858–870. doi:10.1017/S0950268808001222

Litrup E, Torpdahl M, Malorny B, Huehn S, Christensen H, Nielsen EM (2010) Association between phylogeny, virulence potential and serovars of *Salmonella enterica*. Infect Genet Evol 10:1132–1139. doi:10.1016/j.meegid.2010.07.015

Lynne AM, Rhodes-Clark BS, Bliven K, Zhao S, Foley SL (2008) Antimicrobial Resistance genes associated with salmonella enterica serovar newport isolates from food animals. Antimicrob Agents Chemother 52:353–356. doi:10.1128/AAC.00842-07

Malorny B, Hoorfar J, Bunge C, Helmuth R (2003) Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. Appl Environ Microbiol 69:290–296. doi:10.1128/AEM.69.1.290-296

Miller RV, Gammon K, Day MJ (2009) Antibiotic resistance among bacteria isolated from seawater and penguin fecal samples collected near Palmer Station, Antarctica. Can J Microbiol 55:37–45. doi:10.1139/W08-119

Ng LK, Mulvey MR, Martin I, Peters G, Johnson W (1999) Genetic characterization of antimicrobial resistance in Canadian isolates of Salmonella Serovar Typhimurium DT104. Antimicrob Agents Chemother 43:3018–3021

Pan Z, Carter B, Núñez-García J, AbuOun M, Fookes M, Ivens A, Woodward MJ, Anjum MF (2009) Identification of genetic and phenotypic differences associated with prevalent and non-prevalent *Salmonella* Enteritidis phage types: analysis of variation in amino acid transport. Microbiology 155:3200–3213. doi:10.1099/mic.0.029405-0

Rabbia V, Bello-Toledo H, Jiménez S, Quezada M, Domínguez M, Vergara L, Gomez-Fuentes C, Calisto-Ulloa N, González-Acuña D, González-Rocha G (2016) Antibiotic resistance in *Escherichia coli* strains isolated from Antarctic bird feces, water from inside a wastewater treatment plant, and seawater samples collected in the Antarctic Treaty area. Polar Sci 10:123–131. doi:10.1016/j.polar.2016.04.002

Randall LP, Cooles SW, Osborn MK, Piddock LJ, Woodward MJ (2004) Antibiotic resistance genes, integrons and multiple antibiotic resistance in thirty-five serotypes of Salmonella enterica isolated from humans and animals in the UK. J Antimicrob Chemother 53:208–216. doi:10.1093/jac/dkh070

Retamal P, Fresno M, Dougnac C, Gutierrez S, Gornall V, Vidal R, Vernal R, Pujol M, Barreto M, González-Acuña D, Abalos P (2015) Genetic and phenotypic evidence of the *Salmonella enterica* serotype Enteritidis human-animal interface in Chile. Front Microbiol 6:464. doi:10.3389/fmicb.2015.00464

Toro M, Retamal P, Ayers S, Barreto M, Allard M, Brown EW, Gonzalez-Escalona N (2016) Whole-genome sequencing analysis of *Salmonella enterica* serovar enteritidis isolates in Chile provides insights into possible transmission between gulls, poultry, and humans. Appl Environ Microbiol 82:6223–6232. doi:10.1128/AEM.01760-16

Vigo GB, Leotta GA, Caffer MI, Salve A, Binsztein N, Pichel M (2011) Isolation and characterization of *Salmonella enterica* from Antarctic wildlife. Polar Biol 34:675–681. doi:10.1007/s00300-010-0923-8

WHO (2013) Critically Important Antimicrobials for Human Medicine. 4th Revision. http://www.who.int/foodsafety/publications/antimicrobials-fourth/en/. Accessed Jan 2017