Detection of *Salmonella enterica* in Magellanic penguins (*Spheniscus magellanicus*) of Chilean Patagonia: evidences of inter-species transmission

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SUMMARY

Patagonia in southern South America is among the few world regions where direct human impact is still limited but progressively increasing, mainly represented by tourism, farming, fishing and mining activities. The sanitary condition of Patagonian wildlife is unknown, in spite of being critical for the assessment of anthropogenic effects there. The aim of this study was the characterization of *Salmonella enterica* strains isolated from wild colonies of Magellanic penguins (*Spheniscus magellanicus*) located in Magdalena Island and Otway Sound, in Chilean Patagonia. Eight isolates of *Salmonella* were found, belonging to Agona and Enteritidis serotypes, with an infection rate of 0·38%. Resistance to ampicillin, cefotaxime, ceftiofur and tetracycline antimicrobials were detected, and some of these strains showed genotypic similarity with *Salmonella* strains isolated from humans and gulls, suggesting inter-species transmission cycles and strengthening the role of penguins as sanitary sentinels in the Patagonian ecosystem.

Key words: Chile, Patagonia, penguins, *Salmonella enterica*, transmission.

INTRODUCTION

Penguins are long-lived aquatic birds exclusively distributed in the Southern hemisphere and are catalogued as marine sentinels of the ocean's health. This condition has been established, among other reasons, due to their large land breeding colonies [1], and being totally dependent on marine resources [2]. Therefore their population

alterations reflect the regional oceanic variations more accurately and faster than any other aquatic bird [1].

The *Spheniscus* genus includes four species which inhabit the coastal areas from the Pacific and Atlantic Oceans. The Magellanic penguin (*Spheniscus magellanicus*) is distributed in southern South America, including Chile and Argentina. It is the most abundant temperate penguin in the world [1], although with a declining population that has caused its 'Near Threatened' classification by the International Union for the Conservation of Nature [3]. During the reproductive season (spring and summer) it is possible to observe colonies from 30° S in the Pacific and 42° S in the Atlantic

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coasts. In winter, birds migrate to Brazil, South Atlantic Islands, New Zealand and Australia [4].

It has been assumed that emerging infectious diseases (EIDs) constitute an unpredictable phenomenon associated with global changes largely influenced by anthropogenic effects on the environment, hosts and pathogens [5]. In this scenario, penguins are seriously exposed due to resource competition with commercial fisheries and the progressive human invasion of their habitats through population growth and development, including livestock, mining and touristic activities. In addition, the contamination of rivers and oceans with sewage and fishery processing waste predisposes contact with biological agents, threatening the sanitary condition and conservation status of penguins [1]. This situation is particularly important for temperate penguins like *S. magellanicus* in Chilean Patagonia, which are a tourist attraction, increasing the direct and indirect interaction with visitors, domestic animals and their transmissible pathogens.

Worldwide, the need for active surveillance of wildlife-borne pathogens has been recognized, focusing these efforts on prioritized agents. *Salmonella enterica* has been considered within this group, being associated with both water and wildlife [6, 7]. Within the World Health Organization (WHO) Event Management System, salmonellosis is classified as a communicable disease common to humans and animals related to food safety, with transmission through the food chain and water supply [8]. In developed and developing countries, *Salmonella* constitutes an endemic foodborne infection that generates periodic outbreaks in the human population and is considered of great concern for animal health [7, 9, 10]. The most frequent *Salmonella* serovars isolated from wild birds (mainly aquatic birds) are *S. enterica* Typhimurium and *S. enterica* Enteritidis [11–13]. Although in most cases the infection is asymptomatic, these serovars have been associated with disease outbreaks and high mortality [11, 14–16], with reports evidencing a direct transmission of *Salmonella* from wild birds to humans and other animals [17–19].

The aim of this work was to detect *S. enterica* serovars in faecal samples of free-ranging Magellanic penguins from two colonies in Chilean Patagonia. The isolates were characterized by serotyping, antimicrobial susceptibility and genotyping.

MATERIAL AND METHODS

Study area

During January and February in 2012 and 2013, samples from *S. magellanicus* located in Magdalena Island (52° 55′

S, 70° 34′ W) and Otway Sound (52° 58′ S, 71° 13′ W), in southern Chilean Patagonia were collected. For this work, authorization for sampling activities were obtained from the official authority in Magdalena Island and from farm owners in Otway Sound. Additionally, authorizations from Bioethics and Biosecurity Committees of the University of Chile were also obtained.

Sampling

A total of 2114 faecal samples (Table 1) were collected through both environmental and cloacal swabbing. Swabs were placed into Cary–Blair transport medium (Copan, USA) and stored under refrigeration for up to 4 weeks until arrival at the Laboratory of Infectious Diseases, University of Chile, Santiago.

Penguins were captured using a net, following recommendations of Chilean authorities and the Global Penguin Society. In order to avoid breeding interference, only adult animals ranging outside nests were manipulated. Once cloacal swabbing and morphological data collection was completed, the animals were identified by a web tag (National Band and Tag Co. model no. 1005–1) as described previously [20], and then released in the same place as captured.

Bacterial isolates

To isolate bacteria, swabs were placed into 5 ml buffered peptone water (Difco APT broth, Beckton Dickinson, USA) supplemented with 20 µg/ml novobiocin (Sigma, USA) [21], and incubated for 24 h at 37 °C. Then, 100 µl of the suspension was inoculated into modified semi-solid Rappaport–Vassiliadis basal medium (Oxoid, Brazil) supplemented with 20 µg/ml novobiocin and incubated for 24 h or 48 h at 41·5 °C. Cultures with growth were plated onto xylose lysine deoxycholate agar (Difco XLD, Beckton Dickinson) and incubated for 24 h at 37 °C. Suspicious colonies were identified by biochemichal tests and *invA* gene detection by PCR [22]. Next, *S. enterica* strains were serotyped according to the Kauffman–White scheme [23].

For genotypic comparison, a set of *S. enterica* strains isolated from poultry and humans in Chile during 2011 and 2012 were also included in the analysis (Fig. 1). Strains from poultry were provided by the Agriculture and Livestock Service (SAG) and strains from humans by the Institute of Public Health (ISP). A *S. enterica* Agona strain previously isolated from a Kelp gull (*Larus dominicanus*) [24] was also included.

Table 1. *Salmonella strains isolated from Magellanic penguins* (Spheniscus magellanicus)

Location	Year	Isolation rate (%)	Serotype	Strain ID	Antimicrobial resistance
Otway Sound	2012	2/210 (0·95)	Enteritidis	SEN96	Susceptible
			Agona	SAG3	EFT
	2013	4/850 (0·47)	Enteritidis	SEN162SEN163	TE
			Enteritidis	SEN164SEN165	TE
			Enteritidis		TE
			Enteritidis		TE
Magdalena Island	2012	2/436 (0·46)	Agona	SAG1	EFT
			Agona	SAG6	AMP, EFT, CTX
	2013	0/618 (0)	–		–

AMP, Ampicillin; CTX, cefotaxime; EFT, ceftiofur; TE, tetracycline.

Genotyping

Pulsed field gel electrophoresis (PFGE)

PFGE was performed according to the standard protocol recommended by PulseNet (http://www.cdc.gov/pulsenet/pathogens/index.html). Briefly, the digestion was made using *Xba*I (Invitrogen, USA). Electrophoresis was performed using the CHEF DRIII PFGE system (Bio-Rad, USA). The conditions used were 6 V/cm for 21 h at 14 °C with pulse time ranging from 3 to 63 s. As control, *S.* Braenderup H9812 strain was used. The gels were analysed with GEL COMPAR II® software (Applied Maths, Belgium).

Virulotyping

This procedure was made by PCR amplification of *pefA, spvC, sirA, gipA, SEN1417, trhH* and *prot6e* virulence genes, using primers pefA_F (5′-cctgtgacctgaccacttctg-3′), pefA_R (5′-gtaagccactgcgaaagatg-3′), spvC_F (5′-ctccttgcacaaccaaatgcg-3′), spvC_R (5′-tgtctctgcatttcac-caccatc-3′), sirA_F (5′-tgcgcctggtgacaaaactg-3′), sirA_R (5′-actgacttcccaggctacagca-3′), gipA_F (5′-acgactgag-caggctgag-3′), gipA_R (5′-ttggaaatggtgacggtagac-3′), sen1417_F (5′-gatcgctggctggtc-3′), sen1417_R (5′-ctgacc-gtaatggcga-3′), trhH_F(5′-aactggtgccgttgtcattg-3′), trhH_R (5′-gatggtctgtgcttgctgag-3′), prot6e_F (5′-gcctaaggttagtg-tgactctc-3′) and prot6e_R (5′-ctagcagccgttggtatcc-3′). The DNA extraction, reaction mixtures and PCR conditions were developed as described previously [24]. The *S. enterica* Typhimurium ATCC 14028 and *S. enterica* Agona SARB1 strains were used as positive controls.

Antimicrobial resistance phenotypes

Antimicrobial susceptibility was evaluated by the disc diffusion method following CLSI criteria [25].

Antimicrobials tested were (µg/disk) ampicillin (10), amoxicillin–clavulanic acid (20/10), cefotaxime (30), gentamicin (10), trimethoprim–sulfamethoxazole (1·25/23·75), tetracycline (30), ciprofloxacin (5), cefradine (30), ceftiofur (30) and enrofloxacin (10) (Oxoid). *Escherichia coli* ATCC 25922 was used as control strain.

Statistical analyses

Categorical data analyses were made through contingency tables with Infostat (2010v) software (http://www.infostat.com.ar/) using Pearson's correlation coefficient to determine differences ($P < 0.05$).

Results from PFGE and PCR were merged by transforming data in a binary code, using 1 when the character was present (PFGE fragment or PCR gene detection) or 0 when it was absent. The similarity of the strains was calculated according to the Dice coefficient with a 1% tolerance in band position, and the dendogram was constructed using the UPGMA method with TREECON software [26].

RESULTS

Three *S. enterica* Agona and five *S. enterica* Enteritidis strains were isolated, with an overall infection rate of 0·38%. The detection of these serotypes suggest differences according to sampling region and year (Table 1), because Enteritidis strains were only detected in Otway Sound, and Agona strains only in 2012. In addition, phenotypes of resistance against ampicillin, cefotaxime, ceftiofur and tetracycline were detected (Table 1). Resistances to ceftiofur and tetracycline were associated ($P < 0.05$) with Agona and Enteritidis strains, respectively.

The PFGE assay classified the strains in two main clusters, in accordance with their serotypes, with less
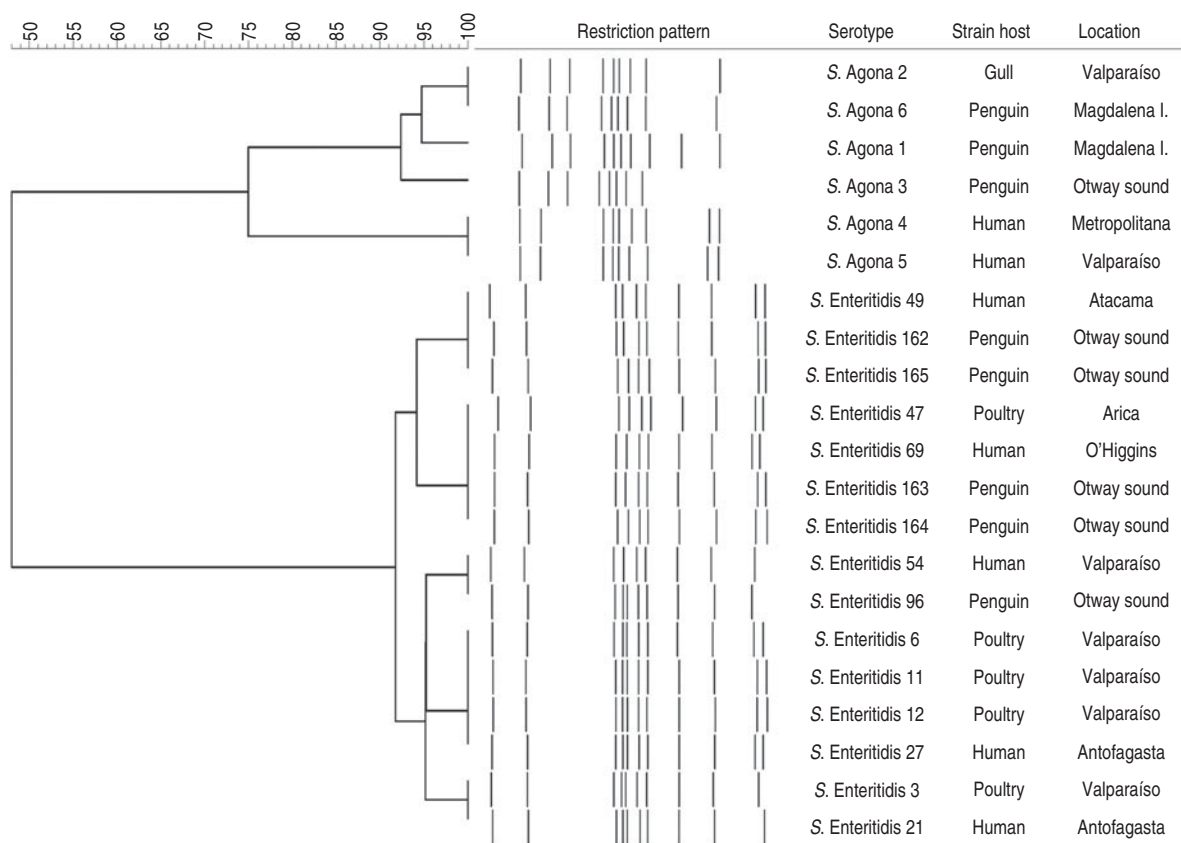
Fig. 1. Dendogram showing genetic similarities (%) between *Salmonella enterica* strains resulting from PFGE assay after digestion with *Xba*I. The tree was constructed using the Dice coefficient and UPGMA algorithm with Gel Compar software (Applied Maths, Belgium).

than 60% similarity between them (Fig. 1). When the PFGE and PCR results were merged (Fig. 2), these major groups were maintained and discrimination improved marginally, with some strains changing from identical patterns to having minor differences (less than 10%) within sub-clusters. Within the two sub-clusters of the *S. enterica* Enteritidis group, all penguin isolates were distinguishable from strains belonging to other hosts. However, similarities were ⩾95%, suggesting genetically close *Salmonella* strains. Within the *S. enterica* Agona group, strains from humans were clearly differentiated from the others, and one penguin's isolate (*S. enterica* Agona 6) had an identical pattern with a gull's strain (*S. enterica* Agona 2). When comparing serotypes, a higher genotypic variability in *S. enterica* Agona strains was found (Figs 1 and 2) compared to the *S. enterica* Enteritidis group.

DISCUSSION

It is now estimated that 70–80% of emerging infectious diseases (EID) in humans have an animal

component in their transmission, and more than half of these have been elicited from wildlife [27]. Moreover, the most common cause of EID in wildlife is the human introduction of pathogens in wild environments [28, 29], among which *S. enterica* is within the group of pathogens that deserve more attention for surveillance [7].

The presence of *Salmonella* in penguins has been reported in *Pygoscelis adeliae* from the Antarctic continent, including serotypes Blockley, Panama and Infantis with an overall infection rate of 13%. Other studies have reported *Salmonella* infection in *P. papua*, with serotypes Enteritidis, Havana and Typhimurium, with isolation rates ranging between 3% and 44% [30, 31]. These works, with an overall 108 positive samples, suggest that *P. papua* could be a major reservoir of the bacterium in Antarctica, contrasting with penguin species *Aptenodytes forsteri* and *Eudyptes chrysolophus* that were also sampled without any detection [32, 33]. In this study we detected a very low occurrence of *Salmonella* infection in *S. magellanicus* (0·38%), with technical, geographical and
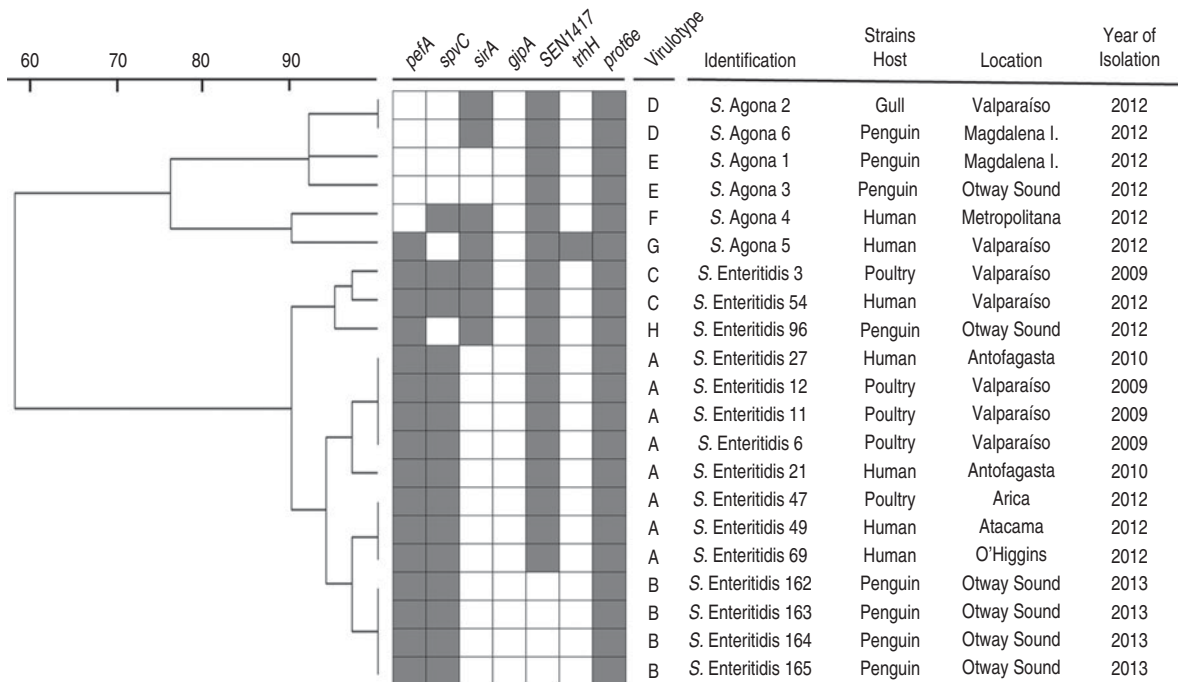
Fig. 2. Dendogram showing genetic similarities (%) between *Salmonella enterica* strains resulting from merged PFGE and PCR data. Detection of virulence-associated genes is depicted as grey squares when present. Virulotypes are indicated by letters according to their frequencies (A:8, B:4, C to E:2, F to H:1). Results from PFGE and PCR were transformed into a binary code, using 1 when the character was present (PFGE fragment or PCR gene detection) and 0 when absent. The tree was constructed using the UPGMA method with TREECON software.

host-related factors that could explain this contrasting result. Nonetheless, reports made from one penguin species cannot be extrapolated to others, which is also supported by a recent description of large variation within the faecal microbiota of different penguin species, a finding that includes bacteria belonging to the family Enterobacteriaceae and several known human pathogens [34].

This is the first report of *Salmonella* infection in free-ranging penguins from South America, and the first description of antimicrobial resistance phenotypes among these isolates. However, a previous work has already shown tetracycline and ampicillin antibiotic resistance in enteric non-pathogenic microorganisms isolated from penguin faecal samples collected near human settlements in Antarctica [35]. This finding, and the fact that tetracycline and ampicillin correspond to widely used antimicrobials in humans, livestock and poultry [36], suggest that these bacterial phenotypes in penguins, which inhabit pristine environments, could be a measure of the anthropogenic effect. However, whether penguins are directly or indirectly being affected by these human footprints is unknown, since other seabirds

in Chile have also been reported to harbour *Salmonella* strains with these and other antimicrobial resistance phenotypes [24]. This hypothetical interspecies transmission gains support with our genotypic results, in which one *S. enterica* Agona strain isolated from a Kelp gull on the Chilean coast, had the same pattern as a penguin isolate (Figs 1 and 2). Furthermore, all *S. enterica* Enteritidis strains isolated from penguins showed high genetic similarity with other human and poultry isolates (Fig. 2), representing additional evidences of close bacterial transmission cycles among hosts.

The analysis based on merged PFGE and PCR results has clearly discriminated bacteria according to their serotypes. The contrasting strains' diversity at the sub-cluster level is in agreement with the relative higher clonality that has been reported for *S. enterica* Enteritidis strains compared to other serotypes [37, 38].

On the other hand, the SEN1417 factor has been identified as a putative ABC transporter protein, which gene is inserted within an unstable chromosomal segment that has been exclusively detected in prevalent phage types isolated from humans and animals [39]. In this study, this sequence was detected in

all *S. enterica* strains isolated during 2012 and none from 2013. Whether this is by coincidence or bacterial evolution remains unknown and should be elucidated in the future.

In Chilean Patagonia, there are wild colonies of *S. magellanicus* neighbouring urban zones with high tourist activity, which increases in the summer during the reproductive period of the animals. It has been calculated that almost 3 00 000 people per year, from all over the world, visit the studied region. However, other activities such as mining, animal husbandry and maritime traffic have been developed near penguins' habitats. The fact that reported Enteritidis and Agona serotypes are among the top five most common zoonotic serotypes isolated from humans in the South American region [10], along with the antimicrobial resistance phenotypes (Table 1) and genotypic similarities detected (Figs 1 and 2), constitute suggestive evidence that penguins have been exposed to human influence in Patagonia. Further efforts are required to characterize the real magnitude of this phenomenon, the temporal and geographical fluctuations of *Salmonella* in penguin colonies, the sanitary effects on these animals, the involvement of other wildlife and the potential for the emergence of new *Salmonella* strains. This knowledge will support decisions to preserve the environment and its wildlife in a globally changing scenario.

DECLARATION OF INTEREST

None.

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