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Genetic and phenotypic evidence of the *Salmonella enterica* serotype Enteritidis human-animal interface in Chile

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Salmonella enterica serotype Enteritidis is a worldwide zoonotic agent that has been recognized as a very important food-borne bacterial pathogen, mainly associated with consumption of poultry products. The aim of this work was to determine genotypic and phenotypic evidence of *S.* Enteritidis transmission among seabirds, poultry and humans in Chile. Genotyping was performed using PCR-based virulotyping, pulse-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). Pathogenicity-associated phenotypes were determined with survival to free radicals, acidic pH, starvation, antimicrobial resistance, and survival within human dendritic cells. As result of PCR and PFGE assays, some isolates from the three hosts showed identical genotypic patterns, and through MLST it was determined that all of them belong to sequence type 11. Phenotypic assays show diversity of bacterial responses among isolates. When results were analyzed according to bacterial host, statistical differences were identified in starvation and dendritic cells survival assays. In addition, isolates from seabirds showed the highest rates of resistance to gentamycin, tetracycline, and ampicillin. Overall, the very close genetic and phenotypic traits shown by isolates from humans, poultry, and seabirds suggest the inter-species transmission of *S.* Enteritidis bacteria between hosts, likely through anthropogenic environmental contamination that determines infection of seabirds with bacteria that are potentially pathogenic for other susceptible organism, including humans.

Keywords: ***Salmonella enterica*, Enteritidis, humans, poultry, seabirds, Chile**

Introduction

Worldwide, reported human *Salmonella* infections are caused by many serotypes, although at present, the highest incidence is represented by *S. enterica* serotype Enteritidis (Hendriksen et al., 2011; Jackson et al., 2013). The changing epidemiology of *S.* Enteritidis infection over the last 20

years has allowed this serotype to become the most prevalent among *S. enterica* serotypes, and is therefore considered an emergent pathogen. Sources of *S.* Enteritidis are generally associated with commercial poultry products, mainly undercooked eggs and meat (Jackson et al., 2013). In Chile, *S.* Enteritidis emerged in 1994, causing food-borne disease in humans and is now included in surveillance systems for both animal and public health services (Fernandez et al., 2003). However, its current endemic condition has shown increasing incidence during the last years, in spite of sanitary regulations and a previous successful campaign against *S.* Typhi (Fica et al., 2012). The epidemiological link between poultry and human *S.* Enteritidis infection has been confirmed through genotypic analysis that has shown the presence of two major *S.* Enteritidis subtypes distributed between both hosts in the Chilean territory (Fernandez et al., 2003). However, because some clinical strains show unique genetic patterns, it seems that infection of humans is also coming from different unknown sources.

Regarding wildlife hosts, seabirds have been associated elsewhere with zoonotic serotypes of *S. enterica*, with evidence that suggests a direct bacterial transmission either among themselves, with other animals or humans (Reche et al., 2003; Pennycott et al., 2006; Dhama et al., 2008; Skov et al., 2008; Horton et al., 2013; Gruszynski et al., 2014). In addition, along the Chilean coast, zoonotic and multi-drug resistant (MDR) *Salmonella* strains have been detected in seashore animals, specifically seabirds and pinnipeds (Lopez-Martin et al., 2011; Sturm et al., 2011; Rodriguez et al., 2012a; Fresno et al., 2013).

The extended geographical distribution, host range and genome plasticity of *S.* Enteritidis have determined genotypic and phenotypic diversity among strains, which contain a striking number of variably detected chromosomal and plasmid genes that may be related to diverse clinical outcomes and adaptive changes favoring survival in different hosts (Pan et al., 2009; Huehn et al., 2010). However, direct correlations between genotypes and phenotypes would not be obvious, since indistinguishable genetic patterns have shown major differences in pathogenicity-associated phenotypes (Yim et al., 2010).

Because of the increasing impact of S. Enteritidis on public health in Chile and the unknown role of wildlife in its epidemiology, the aim of this work was to determine genotypic and phenotypic evidence of *S.* Enteritidis transmission among seabirds, poultry and humans in Chile.

Materials and Methods

Isolates

Ninety *S.* Enteritidis isolates previously isolated from humans, poultry, and seabirds ($n = 30$ each) were analyzed (**Table 1**). Bacteria were grown routinely in liquid culture with Luria-Bertani (LB) medium (Bacto Tryptone, 10 g/L; Bacto Yeast Extract, 5 g/L; NaCl, 5 g/L) adjusted to pH 7 (NaHPO4/NaH2PO4 25 mM), at 37°C for 24 h, with shaking. When necessary, media were solidified by the addition of agar (15 g/L).

Genotypic Assays

Virulotyping

This PCR based test was performed for the identification of genes *invA*, *pefA*, *spvC*, *sirA*, *gipA*, *SEN1417*, *trhH,* and *prot6e*, all of them associated with virulence that have been variably detected in *S.* Enteritidis (Pan et al., 2009; Huehn et al., 2010). After bacterial growth, PCR reactions were performed under standard conditions, as previously described (Fresno et al., 2013).

Pulse-Field Gel Electrophoresis (PFGE)

This assay followed the PulseNet protocol (Ribot et al., 2006). The electrophoresis was performed using CHEF DRIII CHILER (Bio-Rad) equipment. DNA was digested with *Xba*I (50U/sample) endonuclease. PFGE patterns were analyzed with the GEL COMPAR II software (Applied Maths), using the Dice similarity coefficient with a 1% tolerance in band position.

Clustering

Results from virulotyping and PFGE were analyzed through the construction of a binary matrix using "1" for presence and "0" for absence of genes or bands from each isolate. Clusters were determined using via the unweighted pair group method (UPGMA) using TREECON software, and the discriminatory power (DP) was calculated with the Simpson's index of diversity, as reported in a previous study (Hunter and Gaston, 1988).

MultiLocus Sequence Typing (MLST)

This procedure was based on sequencing seven housekeeping genes of *S. enterica* (Achtman et al., 2012), using the primers described in the MLST public database (http://mlst.warwick.ac.uk/mlst/). The sequence type was determined according to the scheme provided on this site.

Phenotypic Assays

Hydrogen Peroxide and Sodium Nitrite Survival Assays

These assays were performed as described by Lu et al. (2002). Briefly, after an overnight growth of bacteria in LB broth the cultures were diluted 1/100 in LB pH 7 or LB pH 5 and challenged with 15 mM H_2O_2 or 10mM $NaNO_2$, respectively. Then, cultures were incubated at 37°C with shaking for 30 min with H_2O_2, or for 3 h with $NaNO_2$. For survival rate analysis, aliquots of culture were plated in triplicate before (time 0) and after challenge (30 min or 3 h). Survival was expressed as the percentage of colony forming units (CFU) after the assay; the count before the challenge was considered as 100%.

Acidic pH Survival

After overnight growth in LB broth, bacteria were washed three times with LB pH 3 (citric acid 0.1 M), diluted 1/100 in the same medium and incubated with shaking at 37°C for 3 h. Survival analysis was performed as with H_2O_2 and $NaNO_2$ assays, with CFU counts at 0 and 3 h.

Starvation Survival Assay

This procedure was based on published reports (Spector and Cubitt, 1992; O'neal et al., 1994), with some modifications.

TABLE 1 | *Salmonella enterica* ser. Enteritidis strains utilized in this study.

Strain ID	Source	Host[a]	Virulence genes								Antimicrobial resistance[b]									
			invA	*pefA*	*spvC*	*sirA*	*gipA*	*SEN1417*	*trhH*	*prot6e*	ENR	AMC	CTX	CN	TE	STX	EFT	CE	AMP	CFR
SEN1	Valparaíso	Poultry																		
SEN2	Valparaíso	Poultry																		
SEN3	Valparaíso	Poultry																		
SEN4	Valparaíso	Poultry																		
SEN5	Valparaíso	Poultry																		
SEN6	Valparaíso	Poultry																		
SEN7	Valparaíso	Poultry																		
SEN8	Valparaíso	Poultry																		
SEN9	Valparaíso	Poultry																		
SEN10	Valparaíso	Poultry																		
SEN11	Valparaíso	Poultry																		
SEN12	Valparaíso	Poultry																		
SEN13	Valparaíso	Poultry																		
SEN14	Valparaíso	Poultry																		
SEN15	Biobío	Kelp gull																		
SEN16	Biobío	Kelp gull																		
SEN17	Biobío	Kelp gull																		
SEN18	Biobío	Kelp gull																		
SEN19	Biobío	Kelp gull																		
SEN20	Biobío	Kelp gull																		
SEN21	Antofagasta	Human																		
SEN22	Metropolitana	Human																		
SEN23	Metropolitana	Human																		
SEN24	Metropolitana	Human																		
SEN25	Arica	Human																		
SEN26	Arica	Human																		
SEN27	Antofagasta	Human																		
SEN28	Coquimbo	Human																		
SEN29	Valparaíso	Human																		
SEN30	Biobío	Human																		
SEN31	Arica	Poultry																		
SEN32	O'Higgins	Poultry																		
SEN46	Arica	Poultry																		
SEN47	Arica	Poultry																		
SEN48	Valparaíso	Human																		
SEN49	Atacama	Human																		
SEN50	Metropolitana	Human																		
SEN51	Metropolitana	Human																		
SEN52	Metropolitana	Human																		
SEN53	Valparaíso	Human																		
SEN54	Valparaíso	Human																		
SEN55	O'Higgins	Human																		
SEN56	Biobío	Human																		
SEN57	Metropolitana	Human																		
SEN58	Metropolitana	Human																		
SEN59	Los Lagos	Human																		
SEN72	Coquimbo	Human																		
SEN73	Magallanes	Human																		
SEN74	Los Lagos	Human																		

(Continued)

TABLE 1 | Continued

Strain ID	Source	Host[a]	Virulence genes								Antimicrobial resistance[b]									
			invA	*pefA*	*spvC*	*sirA*	*gipA*	*SEN1417*	*trhH*	*prot6e*	ENR	AMC	CTX	CN	TE	STX	EFT	CE	AMP	CFR
SEN75	Coquimbo	Human																		
SEN76	Valparaíso	Human																		
SEN77	Valparaíso	Human																		
SEN78	Metropolitana	Human																		
SEN79	Metropolitana	Human																		
SEN80	Metropolitana	Poultry																		
SEN81	Los Ríos	Poultry																		
SEN82	Los Ríos	Poultry																		
SEN83	Arica	Poultry																		
SEN85	Metropolitana	Poultry																		
SEN86	Metropolitana	Poultry																		
SEN87	Metropolitana	Poultry																		
SEN88	Metropolitana	Poultry																		
SEN89	Metropolitana	Poultry																		
SEN90	Metropolitana	Poultry																		
SEN91	Metropolitana	Poultry																		
SEN92	Metropolitana	Poultry																		
SEN95	Arica	Franklin gull																		
SEN96	Magallanes	Penguin																		
SEN97	Coquimbo	Kelp gull																		
SEN98	Coquimbo	Kelp gull																		
SEN99	Coquimbo	Kelp gull																		
SEN100	Coquimbo	Kelp gull																		
SEN101	Coquimbo	Kelp gull																		
SEN102	Coquimbo	Kelp gull																		
SEN103	Coquimbo	Kelp gull																		
SEN104	Coquimbo	Kelp gull																		
SEN105	Coquimbo	Kelp gull																		
SEN106	Coquimbo	Kelp gull																		
SEN107	Coquimbo	Kelp gull																		
SEN108	Valparaíso	Kelp gull																		
SEN109	Valparaíso	Kelp gull																		
SEN110	Valparaíso	Kelp gull																		
SEN111	Valparaíso	Kelp gull																		
SEN112	Valparaíso	Kelp gull																		
SEN113	Valparaíso	Kelp gull																		
SEN114	Valparaíso	Kelp gull																		
SEN115	Valparaíso	Kelp gull																		
SEN116	Valparaíso	Kelp gull																		
SEN117	Valparaíso	Kelp gull																		
SEN118	Valparaíso	Kelp gull																		

Virulence genes and antimicrobial resistance phenotypes appear with shaded squares.

[a]*Poultry, Gallus gallus; Human, Homo sapiens; Kelp gull, Larus dominicanus; Franklin' gull, Leucophaeus pipixcan; Penguin, Spheniscus magellanicus.*

[b]*AMC, Amoxicillin–clavulanic acid; AMP, Ampicillin; CTX, Cefotaxime; CFR, Cefadroxil; CE, Cefradine; EFT, Ceftiofur; ENR, Enrofloxacin; CN, Gentamicin; STX, Trimethoprim–sulfamethoxazol; TE, Tetracycline.*

Briefly, bacterial isolates were inoculated into MOPS-buffered salts (MS) hiPCN (MS, 25 mM KH_2PO_4/K_2HPO_4 pH 7.4, 0.4% glucose, 15 mM NH_4Cl) media, and then incubated for 16–18 h at 37°C. Cultures were washed with distilled water, diluted 1/10 in MS loPCN (MS, 1 mM KH_2PO_4/K_2HPO_4 pH 7.4, 0.2% glucose, 10 mM NH_4Cl) media and incubated at 37°C up to OD_{600} 0.3–0.4. Then, 1 mL of this suspension was washed with distilled water, inoculated into 5 mL MS media and incubated at 37°C for 40 d. Aliquots of culture were plated in triplicate at different times. Survival was expressed as a percentage of CFU in

relation with the maximal CFU count reached between day 0 and 5, which was considered 100%.

Survival within Dendritic Cells (DCs)

For this assay we used nine isolates, three from each host, which showed the highest resistance in the four previous survival assays. For selection, a survival ranking was performed in each assay, assigning 1 to the most susceptible and 90 to the most resistant strain. Then, an average ranking value was calculated for every isolate.

Human peripheral blood mononuclear-derived DCs were obtained from the buffy coats of six healthy donors and prepared as previously described (Vernal et al., 2008). For the infection, day 6 DCs were maintained in culture medium (RPMI-1640 with 10% fetal calf serum) and seeded into tissue culture plates at a concentration of 4×10^5 cells per well. Exponential-phase (OD_{600}, 0.6) grown bacteria were pelleted and suspended in the same medium. Aliquots of bacteria were added to DCs at a multiplicity of infection (MOI) of 50:1. After 1 h of infection, cells were washed three times with PBS, and incubated with cellular culture medium containing gentamicin (200 μg/mL). After additional incubation for 2 and 24 h, DCs were washed with PBS and permeabilized for 30 min with 0.1% Triton X-100, and the titers of intracellular bacteria were determined by serial dilution of cell lysates on LB agar plates. The percentage of survival was calculated at 2 h considering the initial inoculant as 100%, and at 24 h considering the CFU counted at 2 h as 100%.

Ethics Statement

The human DCs protocol included a written consent of all donors, which was approved by the University of Chile Clinical Hospital Scientific Ethics Committee (OAIC Reference #508/11, Exempt Resolution #570).

Antimicrobial Susceptibility

Antimicrobial susceptibility was evaluated by the disk diffusion method following CLSI criteria (CLSI, 2010). Antimicrobials tested were (μg/disk) ampicillin (10), amoxicillin–clavulanic acid (20/10), cefotaxime (30), gentamicin (10), trimethoprim–sulfamethoxazol (1.25/23.75), tetracycline (30), ciprofloxacin (5), cefradine (30), ceftiofur (30), and enrofloxacin (10) (Oxoid®). *Escherichia coli* ATCC 25922 was utilized as a control strain. The multidrug resistance condition was determined by the simultaneous resistance to three or more antimicrobial classes.

Statistical Analysis

Statistical analyses were performed using the ANOVA and Kruskal–Wallis test for independent samples. Categorical data and principal components analyses were performed using data from survival assays and hosts. These tests were calculated using INFOSTAT (2010v) software.

Results

Genotypic Assays

PCR detection of virulence genes from 90 *S.* Enteritidis isolates showed 16 distinct virulotypes, resulting in a low discriminatory power (DP) methodology (0.773) that clustered 80% of isolates within three of these gene combinations (**Table 2**, **Figure S1**). Higher diversity was observed in isolates from poultry and seabirds (9 and 8 virulotypes, respectively) than in isolates detected in humans (5 virulotypes). In addition, virulotypes tended to associate with a specific host ($P < 0.05$), as the most frequent gene combination detected in poultry and human isolates is different from that identified in seabird isolates (virulotypes H and C, respectively. **Table 2**, **Figure S1**). This variation was also observed at genetic level, because *spvC* and *sirA* were differentially detected among hosts ($P < 0.05$), being the first most frequent in bacteria found in humans and poultry and the second in those isolated from seabirds (**Table 3**).

Through the PFGE procedure we obtained fingerprints with 8–12 bands, resulting in 10 major clusters (two or more isolates with identical PFGE profiles) that represent 89% of isolates (**Figure S2**). The DP of this method was 0.891, identifying five identical patterns between isolates from seabirds and humans. However, combined PFGE and PCR results showed the highest DP (0.949), with 16 major clusters that contain 70% of isolates. Six of these are mixed clusters, and two of them contain isolates from humans and seabirds (**Figure 1**). The MLST analyses determined that all *S.* Enteritidis isolates in this study belong to the sequence type (ST) 11.

Phenotypic Assays

In pathogenicity-associated phenotypes there was a significant diversity ($P < 0.05$) of bacterial survival responses among isolates, which was observed in all these assays (data not shown). When results from individual strains were grouped and analyzed according to bacterial host, statistical differences ($P < 0.05$)

TABLE 2 | Virulence gene combinations (Virulotypes) of *Salmonella enterica* ser. Enteritidis strains and their frequency according to host.

Virulotypes		Host (N° of strains)			
ID*	*invA-pefA-spvC-sirA-gipA-SEN1417-trhH-prot6e*	Human (30)	Poultry (30)	Seabirds (30)	Total
O	10000100	0	0	3	3
K	10010001	0	1	0	1
P	10011000	0	1	0	1
G	11000101	0	0	1	1
L	11010001	0	0	2	2
B	11010101	0	0	2	2
F	11100001	0	2	0	2
H	11100101	18	14	2	34
N	11101100	1	0	0	1
D	11101101	0	1	0	1
M	11101110	1	0	0	1
C	11110001	6	4	10	20
A	11110101	4	5	9	18
J	11111000	0	1	0	1
I	11111001	0	1	0	1
E	11111101	0	0	1	1

*Letters were assigned correlatively according to the order in which isolates appear in the dendogram (**Figure S1**).

TABLE 3 | Frequency of virulence associated genes in *Salmonella enterica* ser. Enteritidis strains grouped by host.

Gene	Host[1]			Total *N* (%)
	Human *N* (%)	Poultry *N* (%)	Seabirds *N* (%)	
invA	30 (100)	30 (100)	30 (100)	90 (100)
pefA	30 (100)	28 (93)	27 (90)	85 (94)
spvC	30 (100)[a]	28 (93)[a]	22 (73)[b]	80 (89)
sirA	10 (33)[a]	13 (43)[a]	24 (80)[b]	47 (52)
gipA	2 (7)	4 (13)	1 (3)	7 (8)
SEN1417	24 (80)	20 (67)	18 (60)	62 (69)
trhH	1 (3)	0 (0)	0 (0)	1 (1)
prot6e	28 (93)	28 (93)	27 (90)	83 (92)

[1] *Different letters represents statistical differences between groups ($p < 0.05$).*

were identified in starvation and dendritic cells survival assays (**Table 4**), in which isolates from poultry were the most resistant. Isolates from humans expressed higher resistance to short-term starvation (10 d), but later showed the steepest survival decrease and at 30 and 40 d showed similar CFU counts as bacteria belonging to seabirds. Among the top 10 most resistant isolates in each phenotypic assay, those recovered from poultry were consistently the most frequent (**Figure S3**). Within dendritic cells, isolates from humans were the most susceptible (**Table 4**). The relative survival performance of bacteria analyzed according to their hosts can be graphically seen in **Figure 2**. The poultry isolates have the closest position to most of assays, representing their highest survival capabilities in these challenges. Besides, some phenotypic variables are located forming two groups (A and B, **Figure 2**), depicting a high correlation between them.

Resistance to gentamycin, tetracycline and ampicillin was statistically associated with *S.* Enteritidis isolates from seabirds ($P < 0.05$, **Figure 3**). Additionally, we detected multidrug resistance (simultaneous resistance to three or more different CLSI antimicrobial clasess) in 13 (43%) isolates recovered from seabirds; in contrast, only 3 (10%) and 4 (13%) human and poultry isolates showed multidrug resistance, respectively (**Table 1**).

Significant associations ($P < 0.05$) between genotypic and phenotypic results were detected when isolates from major clusters (A–D in **Figure 1**) were compared (**Table 5**). The cluster A, which only contains isolates from humans and poultry, shows the lowest survival to acidic pH. The cluster C, which is composed by isolates from the three hosts, shows the lowest survival to nitrite-derived free radicals and the highest survival to short-term starvation (**Table 5**).

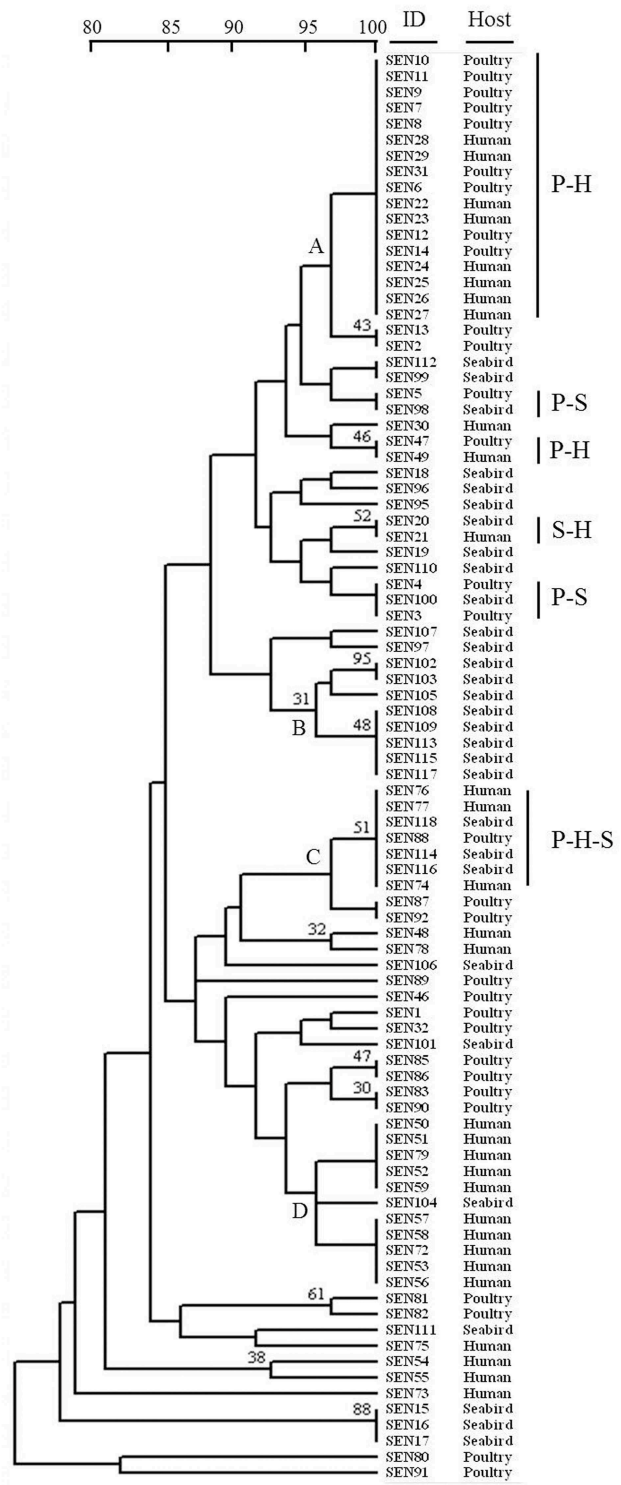

FIGURE 1 | Dendogram showing genetic similarities (%) between *Salmonella enterica* serovar Enteritidis strains resulting from combined PFGE and PCR data. The ID and host are shown for each isolate. Clusters with at least five isolates sharing more than 95% similarity are indicated with letters A, B, C, and D. Mixed clusters are indicated with letters according to hosts (P, poultry; H, human; S, seabird).The tree was constructed using the UPGMA method with the software TREECON.

Discussion

Wild birds have received attention from sanitary authorities because of both their association with several highly transmissible zoonotic pathogens and their ability to disseminate agents with a wide host range, over wide geographical areas (Hubalek, 2004).

TABLE 4 | Average survival percentages (%) of *Salmonella enterica* ser. Enteritidis strains in phenotypic assays grouped by host.

Assay[2]	Host[1]			*P*-value
	Human	Poultry	Seabirds	
pH3	109.43	113.98	116.08	0.70
H_2O_2	19.07	21.09	13.70	0.71
$NaNO_2$	24.29	27.30	19.80	0.85
Starvation 10 d	30.64[a]	23.39[a]	6.19[b]	0.0004
Starvation 20 d	2.31[a]	16.29[a]	0.91[b]	0.0005
Starvation 30 d	0.66[a]	2.73[b]	0.43[a]	0.0022
Starvation 40 d	0.39[a]	1.69[b]	0.31[a]	0.029
DCs invasion	1.4E-05	1.2E-05	4.2E-05	0.66
DCs survival	1.59[a]	27.17[c]	9.49[b]	0.0036

[1] *Different letters represents statistical differences between groups.*
[2] *pH3, H_2O_2, $NaNO_2$ and starvation assays: 30 isolates from every host. Assays with dendritic cells: 3 isolates from every host. Each assay was performed in three independent experiments.*

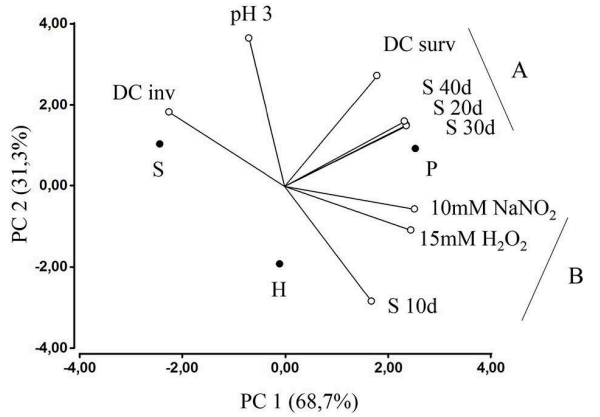

FIGURE 2 | Principal components analysis (PCA) showing the association between *Salmonella enterica* ser. Enteritidis hosts (displayed as filled circles) and results of phenotypic assays (displayed as linear axes and empty circles). In parenthesis appears the variability associated to each principal component (PC). S, seabirds; H, human; P, poultry; DC inv, invasion to dendritic cells; DC surv, survival within dendritic cells; pH3, survival to acidic pH; S, starvation assay (10–40 d, 10 to 40 days); 15 mM H_2O_2, survival to reactive oxygen species; 10 mM $NaNO_2$, survival to reactive nitrogen species.

In this work, *S.* Enteritidis isolates from different hosts have been characterized and compared, attempting to elucidate the human-animal interface of this bacterium in Chile. The existing evidence has established a major transmission chain of *S.* Enteritidis between poultry and humans through food consumption (Fernandez et al., 2003; Fica et al., 2012). However, increasing reports of human cases cannot be solely explained by this link, since prevention efforts have been progressively incorporated in recent years. The use of genotypic and phenotypic methods for bacterial typing was our experimental strategy to shed light on the role of seabirs as a third factor in *Salmonella* epidemiology.

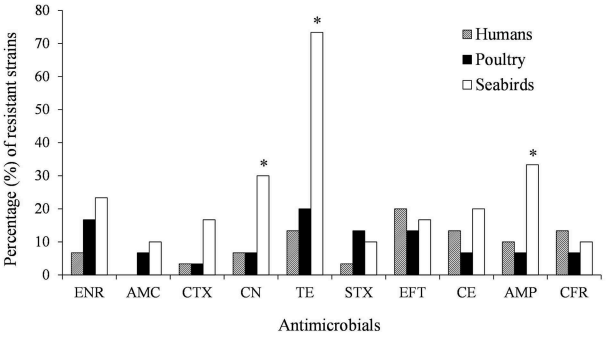

FIGURE 3 | Percentages (%) of antimicrobial resistant *Salmonella enterica* ser. Enteritidis strains grouped according to their hosts. (*$P < 0.05$). AMC, Amoxicillin–clavulanic acid; AMP, Ampicillin; CTX, Cefotaxime; CFR, Cefadroxil; CE, Cefradine; EFT, Ceftiofur; ENR, Enrofloxacin; CN, Gentamicin; STX, Trimethoprim–sulfamethoxazol; TE, Tetracycline.

TABLE 5 | Survival percentages (%) of *Salmonella enterica* ser. Enteritidis genotypic clusters.

Assay	Genotypic cluster[1]				*P*-value
	A	*B*	*C*	*D*	
pH3	76.4[a]	133.6[b]	157.8[b]	132.6[b]	0.0002
H_2O_2	18.8	12.1	11.5	26.3	0.1989
$NaNO_2$	33.9[a]	20.5[ab]	5.5[b]	34.7[a]	0.0094
Starvation 10 d	6.7[a]	6.5[a]	61.4[b]	17.4[a]	<0.0001
Starvation 20 d	2.4	1.0	17.8	1.9	0.1436
Starvation 30 d	2.4	0.5	2.0	0.6	0.1462
Starvation 40 d	1.6	0.2	1.5	0.3	0.2012

[1] *Isolates constituting clusters appear in **Figure 1**. Different letters represents statistical differences between clusters.*

Virulotyping rapidly allows the discrimination of isolates with diverse pathogenic potential or host specificities (Huehn et al., 2010). Despite the low DP of the procedure (0.773), we identified 16 gene combinations heterogeneously distributed among hosts (**Table 2**, **Figure S1**). Variations in the gene repertoire were mainly associated with *spvC* and *sirA* sequences ($P < 0.05$) (**Table 3**). SpvC is a Type III secretion system (T3SS) effector involved in immune evasion and dissemination (Haneda et al., 2012), which importance for the colonization of humans seems represented by our 100% *spvC* detection in isolates from this host (**Table 3**). The *spvC* instability is due to its location on the pSLT virulence plasmid (Fabrega and Vila, 2013), which characterizes the serotypes Enteritidis and Typhimurium. However, the other analyzed pSLT encoded sequences, *pefA* and *prot6e*, were not statistically different among hosts, suggesting that the plasmid gain or loss is not a unique variability mechanism for these sequences. On the other hand, the *sirA* gene encodes a global regulator of virulence, motility and biofilm formation (Teplitski et al., 2006). Its significant association ($P < 0.05$) with bacteria isolated from seabirds suggest particular requirements for *S.* Enteritidis attachment, survival and transmission in marine environments.

Because of its high DP and reproducibility for bacterial typing, the PFGE technique has been utilized to compare *Salmonella* strains isolated from a diversity of hosts and substrates (Ribot et al., 2006; Zheng et al., 2011; Sandt et al., 2013). In this work, the combination of *Xba*I PFGE with virulotyping has allowed the best DP (0.949), comparable to PFGE using a combination of *Xba*I and *Bln*I restriction enzymes (Zou et al., 2012). This procedure is showing several indistinguishable patterns among human, poultry and seabirds isolates (**Figure 1**). Moreover, the MLST analysis has classified all analyzed isolates as belonging to ST11, which is distributed worldwide (http://mlst.warwick.ac.uk/mlst/) and has also been associated with prevalent phage types (Pan et al., 2009),

Overall, genotypic data suggests that wild birds are sharing bacteria, whether directly or indirectly, with poultry and humans, participating in the transmission cycle of *S.* Enteritidis in Chile.

Phenotypic assays were performed in order to determine the pathogenic potential of *Salmonella* isolates from different hosts. The analyzed conditions are mainly found when bacteria face the gastrointestinal lumen upon entering a host via ingestion and within the phagolysosomal environment in phagocytic cells (Behnsen et al., 2015), although survival as free-living bacteria in water, soil or within protozoa in extra-host settings represents a similar challenge (Spector and Kenyon, 2012).

In this work we were able to determine differences among *S.* Enteritidis strains ($P < 0.05$) in all phenotypic assays. When results were grouped according to host source, starvation survival constitutes the unique *in vitro* assay that demonstrates differences ($P < 0.05$) among hosts (**Table 4**), showing that seabird isolates are the most susceptible to nutrient deprivation at 37°C. The human isolates demonstrated a critical downshift between 10 and 30 days (**Table 4**), suggesting a better adaptation to shorter rather than longer periods of starvation. Moreover, poultry strains not only had the highest survival rate to starvation during the entire experiment, but also showed the highest resistance when all *in vitro* assays were considered. Because of that, they are the best represented among the top 10 most resistant strains within every survival assay (**Figure S3**).

Unexpectedly, genotypic clustering at 95% similarity (**Figure 1**) was associated with three survival phenotypes (**Table 5**) in contrast to previous reports with this serotype which, although not using the same methods, did not find such association (Betancor et al., 2009; Yim et al., 2010). The cluster A appear the most defective in pH 3 assay, probably due to absence of seabirds' isolates, which showed the best (non-statistical, $P > 0.05$) fitness in this challenge (**Figure 2**, **Table 4**). Interestingly, the cluster C, which is the unique cluster composed by isolates from the three hosts, is associated with the lowest performance in $NaNO_2$-derived free radicals and the highest survival to a short-term starvation condition (**Table 5**). Whether the transmission between aquatic and terrestrial hosts, suggested by the high genetic similitude of bacteria within cluster C, could be facilitated by such combination of phenotypic responses, is a question that remains to be elucidated.

During the infective process it has been determined that a critical survival challenge facing *S.* Enteritidis inside its host is DCs, because it cannot replicate within these cells as well as in macrophages after invasion (Swart and Hensel, 2012). In order to determine a correlation between *in vitro* phenotypes and survival within human dendritic cells, we performed a gentamicin protection assay using differentiated human peripheral blood monocytes infected with the most resistant *Salmonella* strains isolated from the three hosts. Interestingly, the highest correlation was found with starvation assays at 20 ($r = 0.93$), 30 ($r = 0.92$), and 40 days ($r = 0.94$), suggesting this *in vitro* assay is a predictor of bacterial behavior in the intracellular environment and the pathogenic potential in the human host. In this assay, poultry isolates have again shown the highest survival rate, and unexpectedly, human isolates were the most susceptible (**Table 4**). Consistently, poultry strains have shown the highest survival rates in most assays, suggesting an increased virulence. It is probable that the high contact rates within poultry flocks promote selection of the most rapidly replicating and most virulent clones, because the transmission to other animals will occur no matter the clinical outcome in infected hosts (Berngruber et al., 2013). When represented graphically (**Figure 2**), the relationship between survival to pathogenicity-associated stresses and the source of *Salmonella* isolates highlights the mentioned differential bacterial performances. Furthermore, two groups of assays are formed according to their correlations, one including the short-term starvation survival (S 10 d) with resistance against free radicals (B, **Figure 2**), and the other including long-term starvation survival (S 20, 30, 40 d) with survival within DCs (A, **Figure 2**). This reflects that within each group, the bacterial mechanisms to resist these challenges are sharing stimuli, regulatory factors or effectors, in agreement with previous studies in other bacteria (Watson et al., 1998; Cuny et al., 2005).

The high amount of prescribed antimicrobials in human and veterinary medicine represent a global concern because of the spread of antimicrobial resistant infectious pathogens (WHO, 2014). In general, *S.* Enteritidis isolates have shown low resistance levels against antimicrobials (Huehn et al., 2010; Sandt et al., 2013), contrasting with our results that show host-associated variability in this matter and suggest an overall high frequency of drug resistance and MDR phenotypes, especially in wild birds. From antimicrobials tested, the highest frequencies of resistances were detected against ceftiofur in humans (20%) and against tetracycline in poultry (20%) and wild birds (73%). This constitutes a concerning situation since ceftiofur is prescribed for animal use only, suggesting transmission of resistant strains from animals to humans. Isolates belonging to seabirds expressed significantly higher percentages of resistance ($P < 0.05$) than human and poultry isolates with antimicrobials gentamicin (30%), ampicillin (33%), and tetracycline (73%) (**Figure 3**). The long established environmental persistence of tetracyclines (Hamscher et al., 2002), could explain the high resistance to this antimicrobial. The environment can persistently spread resistant bacteria and sublethal antimicrobial concentrations that, derived from anthropogenic activities (mainly animal farms and wastewater), can select for resistance (Tello et al., 2012; Andersson and Hughes, 2014). Besides, the appearing of virulence-resistance plasmids that encode antimicrobial resistance and virulence factors, determines co-selection of these

functions even in the absence of antimicrobials (Rodriguez et al., 2012b; Gullberg et al., 2014), which could also explain our findings. In any case, these situations represents a potential risk to both public and animal health (Wellington et al., 2013), and justifies the study of these hosts as bio-indicators of resistance traits dispersion into the environment (da Costa et al., 2013).

Altogether, genotypic and phenotypic evidence gathered in this study suggest that *S.* Enteritidis is circulating among wildlife, domestic animals and humans, with human beings participating as incidental (spill-over) hosts. Seabirds can be reservoirs of *Salmonella* strains with potential risk to public and animal health, and could partially explain the progressive rise in the incidence of these serotype-associated outbreaks. Whether such transmission among hosts is direct or indirect is a question that should be addressed in future analyses. Our results support the establishment of biosecurity measures for animal farms and systematic *Salmonella* surveillance campaigns in seabirds, determining not only the genetic similarities of bacterial strains but also their pathogenic potential in susceptible hosts.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00464/abstract

Figure S1 | Dendogram showing genetic similarities (%) between Salmonella enterica ser. Enteritidis strains resulting from PCR data. For each strain, detected genes (black boxes) and host are also shown. The tree was constructed using the UPGMA method with the software TREECON (1000 replicates).

Figure S2 | Dendogram showing genetic similarities (%) between Salmonella enterica ser. Enteritidis strains resulting from PFGE XbaI data. For each strain, the genotypic pattern and host are also shown. The tree was constructed using the UPGMA method with the GEL COMPAR II software (1000 replicates) with a 1% of tolerance in band position.

Figure S3 | Number of Salmonella enterica ser. Enteritidis strains within the top 10 most resistant isolates in every stressful challenge, according to their host source.

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