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Short communication: Characterization of *Salmonella* phages from dairy calves on farms with history of diarrhea

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ABSTRACT

Salmonella enterica can cause disease and mortality in calves. This pathogen is also a zoonosis that can be transmitted by animal contact or by food. The prevalence of *Salmonella* in dairy farms has been reported to range from 0 to 64%, and, due to the diversity of *Salmonella* serovars that can be circulating, *Salmonella* is an important concern for dairy production. Bacteriophages that infect *Salmonella* have been documented to be abundant and widely distributed in the dairy environment. The current study investigated the diversity of *Salmonella* serovars and *Salmonella* phages in 8 dairy farms with a history of diarrhea in southern Chile. A total of 160 samples from sick calves, healthy calves, and the environment were analyzed for *Salmonella* and phage. Isolated phages were characterized and classified by their host range using a panel of 26 *Salmonella* isolates representing 23 serovars. Host ranges were classified according to lysis profiles (LP) and their spatial distribution was mapped. *Salmonella*-infecting phages were identified, but none of the 160 samples were positive for *Salmonella.* A total of 45 phage isolates were obtained from sick calves (11), healthy calves (16), or the environment (18). According to their host range, 19 LP were identified, with LP1 being the most common on all 8 farms; LP1 represents phages that only lyse serogroup D *Salmonella.* The identification of *Salmonella* phages but not *Salmonella* in the same samples could suggest that these phages are controlling *Salmonella* in these farms.
Key words: *Salmonella*, bacteriophage, dairy calf

Short Communication

Salmonella is one of the most common foodborne pathogens in the world, causing an estimated 230,000 deaths yearly (Havelaar et al., 2015). As a zoonotic agent, *Salmonella* is also transmitted by animal contact (Hoelzer et al., 2011a). In cattle, serovars of *Salmonella enterica* ssp. *enterica* have been reported to cause clinical illness (e.g., *Salmonella* Newport, *Salmonella* Dublin) and to colonize asymptomatic carriers (e.g., *Salmonella* Cerro; Rodriguez-Rivera et al., 2014). Prevalence of *Salmonella* in dairy cattle varies; one study of bovine clinical outbreaks in the United States reported a prevalence of fecal shedding ranging from 0 to 53% (Cummings et al., 2010). A recent study reported *Salmonella* isolates in up to 64% of fecal samples from dairy farms in Texas (Rodriguez-Rivera et al., 2016). In Chile, there is a dearth of knowledge about *Salmonella* in dairy farms. One thesis conducted in 2004 in southern Chile reported *Salmonella* in 14% of sampled dairy farms (4/28) (Barrientos, 2005). There are no contemporary publications on the presence of *Salmonella* in dairy farms in Chile, except for one report from surveillance conducted by the Chilean Government that reported the presence of abortions caused by *Salmonella* Dublin in Chile in 2013 (http://www.sag.cl/sites/default/files/situacion_sanitaria_animal_2013_0.pdf).

The most common serovars of *Salmonella* reported in cattle in different parts of the world include Newport, Cerro, Typhimurium, Kentucky, and Dublin (Warnick et al., 2006; Hoelzer et al., 2011b; Rodriguez-Rivera et al., 2014, 2016). Although some of these serovars, such as *Salmonella* Typhimurium, represent a serovar widely distributed in different hosts, other serovars, such as *Salmonella* Dublin, are considered to be better adapted to the bovine host (Nielsen, 2013). *Salmonella* Dublin isolated from cattle has been reported in several studies (Veling et al., 2002; Warnick et al., 2006; Hoelzer et al., 2011b; Nielsen and Dohoo, 2012) and is the serovar most commonly reported in clinical cases of calves, which are highly susceptible between 3 and 6 wk of age (Veling et al., 2002).

The gold standard to detect the presence of *Salmonella* in cattle is culture of bacteria from feces; however, studies have reported that serology has better herd-level

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sensitivity than culturing (Veling et al., 2002). This difference is due, in large part, to the fact that *Salmonella* shedding in the feces is transient, varies over the shedding period, and is affected by antimicrobial treatments (Veling et al., 2002; Nielsen, 2013).

Salmonella bacteriophages appear to be widespread on dairy farms, and the diversity of these phages could reveal interactions between *Salmonella* serovars and specific bacteriophages (Moreno Switt et al., 2013). *Salmonella* phages have been found to be very abundant (up to 97% prevalence) and widely distributed in animal feces, wastewater, and the environment of food animals (Moreno Switt et al., 2013; Pérez Pulido et al., 2016). In addition, studies of *Salmonella* phage host range appear to indicate *Salmonella* serovar specificity. For example, farms with a high prevalence of *Salmonella* Cerro were found to have a predominance of phages specific to *Salmonella* Cerro (Moreno Switt et al., 2013). Another study investigated the host range of *Salmonella* phages isolated from dairy farms in the United States and Thailand and found that US phages showed a narrow host range, whereas Thai phages showed a wide phage host range (Wongsuntornpoj et al., 2014). In this current study, we hypothesized that *Salmonella* phages obtained from calves would have a wide host range, lysing a variety of serovars including *Salmonella* isolates from calves in the same dairy. We characterized *Salmonella* phage dynamics in sick and healthy dairy calves to improve our understanding of the role of phages in controlling *Salmonella* in calves.

Samples were collected from 8 farms located in the Los Ríos and Los Lagos regions of southern Chile. Farms with calves with symptoms of diarrhea were selected based on a target sampling methodology that enrolled farmers willing to take part in a *Salmonella* characterization study. Samples were taken from 10 sick calves, 5 healthy calves, and 5 environmental samples from each farm. Sick calves were selected based on clinical signs and evidence of diarrhea, and healthy calves and environmental samples were collected based on simple random sampling after enumerating all calves and pens using a random number generator in a handheld device. Environmental samples (e.g., holding area, manure storage, and animal beds) were collected in 60-mL sterile containers, using a protocol previously described (Rodriguez-Rivera et al., 2016). Fecal samples were collected from each animal per rectum after a gentle massage of the area. Overall, 160 samples were collected (80 from sick calves, 40 from healthy calves, and 40 from the environment); samples were kept at 4°C and transferred to the laboratory at Universidad Andres Bello (Santiago, Chile) for processing within 24 h.

All samples were cultured for *Salmonella*. Briefly, 10 g of each sample was enriched in 90 mL of peptone water (Becton-Dickinson, Franklin Lakes, NJ) and incubated at 37°C overnight. Two subsets were then incubated: one set of 1 mL of sample in 9 mL of tetrathionate broth (Becton-Dickinson) with iodine and the other set of 100 μL of sample in 9.9 mL of Rappaport Vassiliadis broth (Becton-Dickinson) with novobiocin (20 μg/mL). Finally, aliquots of 100 μL were inoculated onto XLT4 agar with XLT4 supplement (xylose lactose tergitol; Becton-Dickinson). Suspect colonies were confirmed by *invA* PCR (Kim et al., 2007). To validate the isolation methods, random samples were processed in the Laboratory of Food at Universidad Austral (Campus Isla Teja, Valdivia, Chile), which is ISO-certified for *Salmonella* detection.

To isolate phages, equal proportions of all samples from a single source on each farm were combined; specifically, samples from sick calves were pooled, samples from healthy calves were pooled, and environmental samples were pooled, forming 3 pooled samples per farm. These pooled samples were used to isolate and purify *Salmonella* phages, as previously described (Moreno Switt et al., 2013). Four *Salmonella* strains,

Table 1. Summary of phages detected and purified from dairy calves in this study

			Number of *Salmonella* phages isolated[1]			
Farm ID	Sampling date	Total isolated phages	Enteritidis FSL S5-371	Heidelberg FSL S5-455	Typhimurium FSL S5-370	Infantis FSL S5-506
1	April 27, 2015	2	2 (1/1/0)	0	0	0
2	April 28, 2015	8	3 (1/1/1)	4 (0/2/2)	1 (0/0/1)	0
3	April 29, 2015	6	3 (1/1/1)	0	3 (1/1/1)	0
4	May 28, 2015	3	3 (1/2/0)	0	0	0
5	May 28, 2015	7	6 (2/2/2)	1 (0/0/1)	0	0
6	May 29, 2015	9	4 (2/0/2)	2 (2/0/0)	0	3 (0/2/1)
7	May 29, 2015	6	4 (0/2/2)	2 (0/0/2)	0	0
8	August 26, 2015	4	4 (0/2/2)	0	0	0
Total		45	29	9	4	3

[1]Number of phages displaying clear plaques that were identified and purified in the strain representing the 4 *Salmonella* serovars (numbers in parentheses indicate samples from sick calves/healthy calves/environment). FSL = Food Safety Laboratory at Cornell University (Ithaca, NY).

previously characterized by the Food Safety Laboratory (**FSL**) at Cornell University (Ithaca, NY), were used as host for phage isolation: Infantis (FSL S5-506), Heidelberg (FSL S5-455), Typhimurium (FSL S5-370), and Enteritidis (FSL S5-371). The host range of the isolated phages was characterized using a panel of 26 *Salmonella* strains using previously described protocols (Vongkamjan et al., 2012; Moreno Switt et al., 2013). In addition, one isolate of *Escherichia coli* and one isolate of *Pseudomonas* spp. were added. Host ranges were

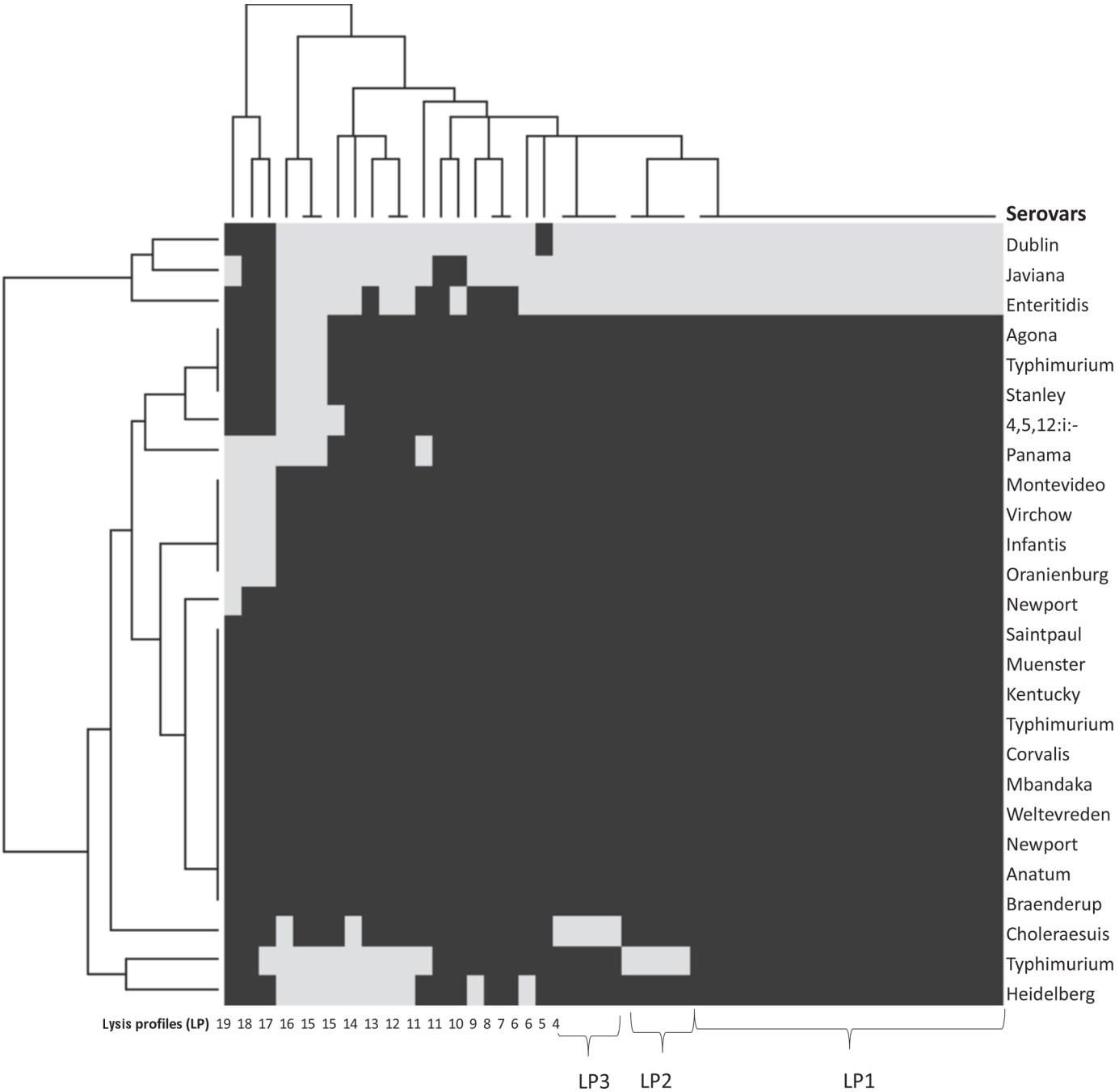

Figure 1. Heatmap representing phage relatedness according to the phage lysis profiles. A hierarchical clustering was conducted using the Ward's method of binary distance with the software R (version 2.10.0; R Development Core Team, Vienna, Austria; http://www.R-project.org). In the x-axis are the lysis profiles (LP) and in the y-axis are the serovars used to characterize the phages identified in this study. Gray represents lysis and black represents no lysis.

classified by lysis profiles (**LP**). Results were mapped using spatial software (ArcGIs v.10, Esri, Redlands, CA).

This study searched for *Salmonella* and *Salmonella* phages in dairy farms with history of calves with diarrhea; *Salmonella* was not identified in any of the 160 samples tested. Importantly, in the sampled farms, antimicrobial treatment is generally used to treat diarrhea, but treatments are poorly recorded; therefore, it is possible that these antimicrobial treatments inhibited *Salmonella* growth, explaining our results.

In this study, we identified an abundance of bacteriophages in sick calves (11 phages), healthy calves (16 phages), and the environment (18 phages) of all 8 sampled dairy farms (Table 1), with significantly more bacteriophages being isolated from healthy calves than from sick calves ($\chi^2 = 11.933$, $P < 0.05$). Previous studies have also reported an abundance of phages in dairy farms in different parts of the world with varied productions systems (i.e., intensive or backyard; Moreno Switt et al., 2013; Wongsuntornpoj et al., 2014).

This study detected and purified 45 phages; the majority were isolated with the *Salmonella* Enteritidis host (64.4%), followed by *Salmonella* Heidelberg (20%), *Salmonella* Typhimurium (8.9%), and *Salmonella* Infantis (6.7%) (Table 1). In previous studies in the United States and Thailand, phages from dairy farms were mostly isolated using serovars Weltevreden, Newport, Enteritidis, Typhimurium, and Cerro (Moreno Switt et al., 2013; Wongsuntornpoj et al., 2014). Although a previous study reported that a high prevalence of *Salmonella* Cerro was associated with the isolation of *Salmonella* phages infecting this serovar, *Salmonella* Cerro was not detected in this study. *Salmonella* Cerro has been described as being adapted to the bovine intestine (Hoelzer et al., 2011b; Rodriguez-Rivera et al., 2014). Our findings, along with previous reports, indicate an association between *Salmonella* serovars and phages capable of infecting these serovars on the same farm.

The host range characterization identified 19 LP (Figure 1; Supplemental Table S1; https://doi.org/10.3168/jds.2016-11569) that were widely distributed among the farms (Supplemental Figure S1; https://doi.org/10.3168/jds.2016-11569). Among all LP, LP1 was the most frequent and widely distributed (18 phages from all 8 farms); these profiles included phages that lysed *Salmonella* Enteritidis, *Salmonella* Javiana, and *Salmonella* Dublin, all serogroup D *Salmonella.* Importantly, LP1 was found in farms up to 120 miles apart (Supplemental Figure S1). In addition, these 3 serovars were found to be the most susceptible to phage lysis in this study (Figure 1). Previously, our group identified serogroup D-specific phages in other regions of Chile, suggesting a very wide distribution of phages infecting serogroup D *Salmonella* (our unpublished data).

To determine if the isolated phages could infect other bacteria, we tested whether isolated phages could lyse isolates of *E. coli* and *Pseudomonas* spp. None of the phages lysed these bacteria. Although some *Salmonella* phages have been reported to lyse *E. coli* (Moreno Switt et al., 2013), phages obtained from calves in this study appear to be specific to *Salmonella*. Overall, results from this study indicate that *Salmonella* phages are abundant and widely distributed in sick and healthy dairy calves; it is tempting, therefore, to speculate that these widely distributed phages might be controlling *Salmonella* in the feces of these animals.

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