



Detection of Antimicrobial Resistant *Salmonella enterica* Strains in Larval and Adult Forms of Lesser Mealworm (*Alphitobius diaperinus*) From Industrial Poultry Farms

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The lesser mealworms (*Alphitobius diaperinus*) constitute a common cosmopolitan pest in poultry flocks and may colonize the litter in adult and larval forms. Previous studies have documented their potential as carriers of enteric pathogens. In this context, *S. enterica* constitutes a prioritized zoonotic agent in the poultry industry due to the sanitary risks and economic losses associated with its presence. The aim of this study is to describe the presence of *S. enterica* strains in larval and adult forms of *A. diaperinus* collected from poultry litter belonging to industrial farms located in the central zone of Chile. A total of 403 specimens (203 adults and 200 larvae) were sampled from three farms and 25 flocks. For bacteriological isolation, beetles were processed to differentiate external and internal contamination. Then, isolates were serotyped according to the Kauffman-White scheme and antimicrobial resistance phenotypes were determined using the disk diffusion method. Gene sequences from the megaplasmid pESI were identified through a PCR based test. These procedures led to the detection of 15 *S. enterica* isolates, belonging to serotypes Infantis (14) and Livingstone (1), from both adults (6) and larval (9) specimens, with a similar external (7) and internal (8) distribution. Furthermore, all *S. Infantis* isolates showed antimicrobial resistance and evidence of megaplasmid pESI carriage, with all possessing multidrug-resistant phenotypes. Our results confirm that *A. diaperinus* constitutes a potential reservoir of zoonotic *Salmonella* strains of sanitary and economic concern for the industry and for public health.

Keywords: poultry, Chile, *Salmonella*, *Alphitobius diaperinus*, drug resistance

INTRODUCTION

The lesser mealworms (*Alphitobius diaperinus*) constitute a common pest in poultry flocks (1), characterized as a scavenger arthropod which colonizes the litter in adult and larval forms. They are able to survive within flocks by consuming feces, food and dead birds, but can also affect residential areas in close proximity to fields treated with manure (2). This insect has been reported to serve as a vector for several enteropathogens, including *E. coli*, *Campylobacter*, and *Salmonella enterica*, among others (3).

S. enterica is an enteric pathogen that is widely distributed in nature and produces a variety of diseases in a range of hosts, including humans, mammals, birds, and reptiles. In addition, insects, plants, and unicellular organisms may also harbor bacteria in the environment (4), leading to the ubiquity and persistence of these bacteria in infecting hosts. More than 2,600 serotypes within the *S. enterica* species have been described, including both host-restricted and host-generalist serotypes (5).

The zoonotic risk of *Salmonella* is mainly associated with its transmission through consumption of contaminated animal and plant-derived foods (6). This usually results in a self-limiting gastroenteritis, although patients with some risk factors, such as infants, immunocompromised individuals and the elderly, can develop extra-intestinal infections that can cause meningitis, sepsis, and even death. In recent years, infection with this bacterium in humans has been among the most common causes of notifiable outbreaks (7). Globally, *S. enterica* serotype Enteritidis (*S. Enteritidis*) and *S. Typhimurium* represent the most common serotypes that cause disease in humans (8). However, several other bacteria, which may be classified as emergent clones or serotypes, have also been responsible for outbreaks in recent years (9, 10). In Chile, the public health service has developed food chain surveillance and food-associated outbreak investigations programs. Together these activities have established *Salmonella* as the most common pathogen involved in foodborne disease outbreaks with Enteritidis, Infantis, and Typhimurium the most frequently detected serotypes (11). Furthermore, the official veterinary service controls a biosafety program in the poultry industry, with specific indications for prevention and early notification of biological agents, and an official microbiological control program for exported animal products (12).

The progressive increase of antimicrobial resistant bacteria presents a current menace (13), and is cataloged by WHO as one of the most important global threats to public health. For this reason, antimicrobials have been categorized and prioritized in order to preserve their effectiveness (14). In recent years, a gradual increase in drug resistant *Salmonella* strains has been documented in the human food chain, leading to more serious clinical cases and more hospitalizations (15). The purpose of this study is to report and characterize the isolation of antimicrobial-resistant *Salmonella* serotypes in larval and adult forms of *A. diaperinus* collected from poultry litter belonging to industrial farms located in the central zone of Chile.

METHODS

Samples

During December 2018, a total of 403 specimens (203 adults and 200 larvae) were sampled from 25 flocks belonging to three industrial farms located in the central zone of Chile. The insects were collected independently from manure and then stored in sterile 10 mL tubes.

Bacteriological Isolation and Serotyping

Once at the lab, each sample was processed using a two-step procedure for bacteriological isolation in order to differentiate

between external and internal contamination. In the first step, the insects were immersed for 10 s in 5 mL of sterile buffered peptone water (Difco BPW broth, Beckton Dickinson, Franklin Lakes, NJ, USA) supplemented with 20 µg/mL of novobiocin (Sigma, St. Louis, MO, USA). In the second step, insects were recovered with tweezers and immersed for 1 min in 95% ethanol, air dried and washed with PBS, and homogenized in 1.5 mL tubes using plastic stems. Insect remains were then inoculated into 5 mL of sterile BPW broth supplemented with 20 µg/mL novobiocin. Each inoculate was incubated for 24 h at 37°C. Then 100 µL of each suspension was inoculated into modified semisolid Rappaport Vassiliadis basal medium (Oxoid, Sao Paulo, Brazil) supplemented with 20 µg/mL of novobiocin and incubated at 45.1°C for either 24 or 48 h, depending on whether or not bacterial growth was observed. Cultures were plated onto Xilose Lysine Deoxicholate agar (Difco XLD, broth, Beckton Dickinson, Franklin Lakes, NJ, USA) and suspicious colonies were identified using biochemical tests and *invA* gene detection by PCR (16), using the *S. Enteritidis* SARB 16 as a control strain. Finally, *S. enterica* isolates were serotyped according to the Kauffman-White scheme (5).

Disk Diffusion Method

Antimicrobial resistance phenotypes were determined by the disk diffusion method according to the standards recommended by the Clinical Laboratory Standards Institute (17). The following antimicrobials were evaluated: ampicillin (10 µg), amoxicillin + clavulanic acid (20/10 µg), ceftiofur (30 µg), ceftazidime (30 µg); ceftriaxone (30 µg), cefadroxil (30 µg), gentamicin (10 µg), streptomycin (10 µg), azithromycin (15 µg), tetracycline (30 µg), ciprofloxacin (5 µg), enrofloxacin (10 µg), nalidixic acid (30 µg), sulfamethoxazole + trimethoprim (20/5 µg), sulfisoxazole (10 µg), chloramphenicol (30 µg), and fosfomicin (20 µg). *Escherichia coli* ATCC 25922 was used as a control strain. The multi-drug resistance (MDR) condition was determined by the simultaneous resistance to three or more antimicrobial classes (18).

PCR Assays

After bacterial growth was observed, nucleic acids were extracted using the DNA extraction kit (Roche®) according to the manufacturer's instructions. Then, a PCR based test was performed under standard conditions for the identification of the pESI (plasmid for emerging *S. Infantis*) genes *faeAB*, *ipfA*, *merA*, *pemK*, *ccdAB*, and *traC*, using primers described previously (19).

Statistical Analyses

Sampling variables such as the presence of *Salmonella* in poultry flocks, the bacterial location in the *A. diaperinus* body and its developmental stage, were contrasted with isolation results through a logistic regression analysis, using the INFostat (2010v) software.

RESULTS

Out of the samples analyzed, 10 flocks belonging to the three farms were found to be infected. A total of 15 *S. enterica* isolates

were detected from lesser mealworms, including *S. Infantis* (14) and *S. Livingstone* (1) serotypes. Additionally, all *S. Infantis* isolates showed multi-drug resistance phenotypes, and the pESI sequences were variably detected in most of the strains, with the exception of *S. Livingstone* (Table 1).

Analysis of infection status of flocks, bacterial location and the stage of host development variables, determined that none of them were statistically associated with *Salmonella* detection ($p > 0.05$).

DISCUSSION

Within poultry farms, *S. enterica* contaminates productive units and the food chain through diverse transmission pathways, including environmental sources (20) that can hold bacteria, allowing repeated infection of hosts. In fact, when such reservoirs remain unnoticed, continuous exposure and outbreaks over several years have been documented, both in animal and human settings (21, 22). This study suggests that one such potential reservoir are lesser mealworms, which are common arthropods that live on the manure within poultry facilities (23). On sampled insects, bacterial isolates were indistinctly detected both externally and internally and in larval and adult forms ($p > 0.05$). Although larvae may have a higher capacity to transmit infection to chickens than do the adult forms (23), our results suggest that beetles always present a risk of carrying and spreading *Salmonella* within poultry flock environments, that apparently depends on the bacterial dose to which these animals are exposed (24). It is feasible that *A. diaperinus* directly and indirectly transmits *Salmonella* to animals, since it is consumed by broiler chicks (25, 26) and also disseminates bacteria to the chicken manure (24).

Whether *A. diaperinus* is a reservoir host or simply a mechanical vector of *Salmonella* is an still unknown condition that new studies should address. Whatever the role, the field evidence suggests that this arthropod can survive cleaning and disinfection procedures, which presents a risk for its transmission in poultry pens (24, 27, 28). Furthermore, this insect represents a good protein source for human consumption (29), resulting in an additional public health risk if zoonotic pathogens colonize its body from the environment or through its diet (29).

It has been determined that beetles harboring *Salmonella* in their gut can shed bacteria thorough their feces for an average of 8 days, allowing persistent pathogen dispersal between flock rotations (30). In the sampled farms, routine biosecurity management practices currently incorporate an exhaustive cleaning procedure in which manure and organic matter are removed with pressurized water, and a sanitation procedure in which disinfectants are applied to pens during 14-days empty periods. Despite of these procedures, beetles have not been eradicated and persist in consecutive flocks, as does *S. enterica*, suggesting that insects play a role in the continuous exposure of birds to this bacterium. Personnel from the farms recognize the presence of small cracks and crevices within facilities, in which arthropods may survive and continuously contaminate the surrounding environment.

Regular surveillance is performed by the same farms to detect *Salmonella* infection in poultry and flocks are classified according to their infection status with this bacterium. However, in this study such condition was not a predictor of *Salmonella* detection in beetles ($p > 0.05$), suggesting that a more stringent surveillance sampling is needed, or that a differential risk exists in contamination of arthropods and chickens within pens. In fact, it has been reported that insects may be early indicators of *Salmonella* infection in flocks, with higher detection rates than other samples obtained from these environments (27).

In analyzed specimens, *S. Infantis* and *S. Livingstone* serotypes were detected. *S. Infantis* is an emerging serotype within the poultry industry, which apparently emerged 75 years ago and then expanded globally during industrialization of livestock production (31). *S. Livingstone* is a wide host range serotype associated with diverse hosts, including cattle (32), pigs (33), poultry (34), and sea lions (35), among others, although it is less frequently linked to disease in humans than is *S. Infantis* (8). The lower frequency of this serotype (and the absence of others) in sampled beetles may be explained by a competitive exclusion phenomenon that characterizes the transmission and colonization of *Salmonella* in poultry (36) and inside the gut of *A. diaperinus* (29).

Despite of causing milder clinical outcomes in humans than other serotypes (37), emergent *S. Infantis* strains have been associated with the acquisition of chromosomal mutations and the transmission of genetic traits, such as plasmids, which confer MDR phenotypes in most of the strains recently isolated from poultry around the globe (38–40). In Chile, antibiotics used in animals account for 95% of all antibiotics imported by the country (41), suggesting that the practice of veterinary medicine could have major impacts on the selection of drug resistant bacteria (15). In 2018, the surveillance of non-typhoidal *Salmonella* carried out by the public health service reported the emergence of *S. Infantis*, as the second most frequent serotype in both intestinal and extraintestinal clinical cases, after *S. Enteritidis*. Moreover, in the same year *S. Infantis* showed the highest antimicrobial resistance levels against sulfamethoxazole /trimethoprim, chloramphenicol and ampicillin, with resistance levels ranging between 48 and 58%. In contrast, high susceptibility to ciprofloxacin was still observed, although some extraintestinal isolates (3/18) expressed resistance against this drug (11). In general, such results agree with the phenotypes observed in this study, suggesting that strains detected in lesser mealworms have been subjected to similar selection pressure within poultry environments and belong to the same transmission chains that cause disease in humans.

The *S. Infantis* drug resistance has been associated with the unique pESI megaplasmid which was initially described in Israeli isolates in 2007 (42), and along with with some pESI-like variants, has since been described in other territories across the world (43–45). This mobile genetic structure can be transferred to other commensal or pathogenic bacteria within the host intestinal environments (19). Although contains conserved and polymorphic segments, the plasmid-associated pattern of resistance includes antimicrobials such as tetracycline, sulfamethoxazole and trimethoprim, among others (19, 43), which

TABLE 1 | Description of *Salmonella* isolates detected in lesser mealworms.

Salmonella serotype	Host data*			Antimicrobial resistance**													pESI genes										
	ID	Loc	Stage	AMP***	AMC***	EFT***	CAZ	CRO***	CFR	CN***	S	AZN***	TE	CIP***	ENR***	NA***	STX	SF	C	FOT***	faeAB	ipfA	merA	pemK	ccdAB	traC	
Infantis	3	Int	Adult																								
Infantis	38	Ext	Adult	■		■		■				■															
Infantis	50	Ext	Adult											■													
Infantis	82	Int	Adult																	■							
Infantis	90	Int	Larva	■		■		■				■						■				■					
Infantis	100	Int	Larva	■		■		■				■										■					
Infantis	102	Int	Larva	■		■		■				■										■					
Infantis	124	Ext	Larva	■		■		■				■										■					
Infantis	126	Ext	Larva	■		■		■				■										■					
Infantis	126	Int	Larva	■		■		■				■										■					
Infantis	268	Ext	Larva	■		■		■				■										■					
Infantis	294	Ext	Adult	■		■		■				■										■					
Infantis	394	Int	Adult	■		■		■				■										■					
Infantis	403	Int	Larva	■		■		■				■										■					
Livingstone	356	Ext	Larva	■		■		■				■										■					

*ID, identification; Loc, location; Int, Internal; Ext, External. Gray spaces represent a phenotype or gene detection.

**AMP, Ampicillin; AMC, Amoxicillin/ Clavulanic acid; EFT, Ceftiofur; CAZ, Ceftazidime, CRO, Ceftriaxone; CFR, Cefadroxil; CN, Gentamicin; S, Streptomycin; AZN, Azithromycin; TE, Tetracycline; CIP, Ciprofloxacin; ENR, enrofloxacin; NA, Nalidixic acid; SXT, Sulfamethoxazole /Trimethoprim; SF, Sulfisoxazole; C, Chloramphenicol; FOT, Fosfomicin.

***Critically important antimicrobials (14).

have been subjected to positive selection and spreading of drug resistance as a result of widespread and common use (31). In this study, we found resistances against these same and other antimicrobials, as well as genetic evidence of the pESI presence, with some polymorphisms among bacterial isolates (Table 1). The existence of is plasmid might explain the MDR phenotype observed in all *S. Infantis* isolates, which is likely dispersed within local productive farms. Furthermore, these MDR phenotypes have also been associated with enhanced resistance to heavy metals and environmental fitness of the strains in which they are present (46). These characteristics represent bacterial survival mechanisms that challenge the strategies implemented by producers and sanitary authorities to control and prevent salmonellosis. The emergence of *S. Infantis* strains harboring pESI or pESI-like plasmids is a risk to public health and requires exhaustive epidemiological characterizations of the animal and environmental transmission chains so that effective control methods can be implemented.

This study has some limitations. All samples were taken during a single month and belong to industrial farms from the same company, and therefore may not be representative of the epidemiological conditions of other seasons, environments or farms throughout the country. A more extended sampling scheme, involving the collection of samples over a longer period of time and from a wider variety of companies in Chile, would have resulted in a more complete understanding of the *S. Infantis*-*A. diaperinus* relationship. In addition, a higher resolution method is needed for the plasmid description, in order to characterize and compare the pESI structure of Chilean isolates

with those reported elsewhere, and for to elucidate virulence functions and risk potentials of these strains. A strength of this study is that the isolation procedure was able to discriminate between internal and external *Salmonella* contamination in adults and larvae from *A. diaperinus*, confirming the ability of this insect for bacterial transmission within flock environments.

In conclusion, there are MDR *Salmonella* strains in lesser mealworms within industrial poultry farms from Chile. These arthropods constitute a host reservoir of this zoonotic pathogen and represent economic and sanitary risks to the food chain of the country. In this regard, this study supports actions for permanent control strategies of *A. diaperinus* populations in animal facilities.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AD: field sampling, laboratory work, and analyses of results. NP: field sampling, analysis of results, and writing the manuscript. PR: conception, design of the study, analysis, interpretation of results, and writing the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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