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Antibiotic-resistant Salmonella Enteritidis isolated from egg contents of hens housed in conventional cages of technified farms in Sinaloa, México

Salmonella Enteritidis resistente a los antibióticos aislada del contenido de huevo de gallinas alojadas en jaulas convencionales de granjas tecnificadas en Sinaloa, México

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ABSTRACT

Antimicrobial resistance in Salmonella is a threat to humanity. Salmonellosis is the main foodborne zoonosis caused by egg consumption and the second zoonosis with the most hospitalizations and deaths. Chickens are its main reservoir and it is estimated that 40.2 % are carriers. The objective of this work was to determine the presence of antibiotic-resistant Salmonella Enteritidis strains in the egg content of chickens housed in conventional cages on technified farms in Sinaloa, Mexico. The isolation was on Hektoen enteric agar and XLD, the identification of S. Enteritidis was by biochemical tests and PCR, and the determination of antimicrobial resistance was by the disk diffusion method. 0.625 % (2/320) of eggs tested positive for Salmonella Enteritidis, both strains resistant to beta-lactams and quinolones and 1 of them also presented resistance to nitrofurans (multi-resistant). This is the first report of S. Enteritidis in egg contents on farms in Sinaloa. This reflects the presence of Salmonella in hens, which could put the egg consuming population of Sinaloa at risk of zoonosis. In addition, the resistance of these bacteria to antibiotics indicates vulnerability to medical treatments.

KEY WORDS: Salmonella, Enteritidis, hens, egg, resistance, multi-resistance, antibiotics, safety, food.

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RESUMEN

La resistencia antimicrobiana en Salmonella es una amenaza para la humanidad, la salmonelosis es la principal zoonosis por transmisión alimentaria por consumo de huevo y segunda zoonosis con más hospitalizaciones y muertes, las gallinas su principal reservorio y se estima que el 40.2 % son portadoras. El objetivo de este trabajo fue determinar la presencia de cepas de Salmonella Enteritidis resistentes a los antibióticos en el contenido de huevo de gallinas alojadas en jaulas convencionales de granjas tecnificadas en Sinaloa, México. El aislamiento fue en agar entérico Hektoen y XLD, la identificación de S. Enteritidis fue mediante pruebas bioquímicas y PCR, y la determinación de la resistencia antimicrobiana por el método de difusión en disco. El 0.625 % (2/320) de huevos resultaron positivos a Salmonella Enteritidis, ambas cepas resistentes a betalactámicos y quinolonas y 1 de ellas presentó además resistencia a nitrofuranos (multirresistente). Este es el primer reporte de S. Enteritidis en el contenido del huevo en granjas de Sinaloa, esto refleja la presencia de Salmonella en las gallinas, lo cual podría poner en riesgo de zoonosis a la población consumidora de huevo de Sinaloa, además, la resistencia de estas bacterias a antibióticos indica la vulnerabilidad a los tratamientos médicos.

PALABRAS CLAVE: Salmonella, Enteritidis, gallinas, huevo, resistencia, multirresistencia, antibióticos, inocuidad, alimentos.

Introduction

The transmission and proliferation of antimicrobial-resistant bacteria (AMR) through the food chain contribute to the widespread dissemination of these microorganisms, making AMR one of the greatest modern threats to humanity. Bacteria of the Salmonella genus are the major cause of foodborne gastroenteritis (salmonellosis) worldwide (Giancoboni, 2023; Castro-Vargas et al., 2021; Coelho et al., 2020). In Europe, it is responsible for 30.7 % of all foodborne diseases and ranks as the second zoonosis with the highest number of hospitalizations and deaths (Cardoso et al., 2020). Due to the increasing threat of AMR, the WHO has classified Salmonella as a high priority pathogen (WHO, 2017).

Globally, approximately 80.3 million cases of salmonellosis and 155,000 related deaths are reported annually (Saenz et al., 2022), with domestic birds being the primary reservoir of Salmonella spp. The genus comprises more than 2,600 serovars (Amado, 2023; CDC, 2013), with S. Enteritidis being the most frequently isolated serovar in human salmonellosis cases. In Europe and the United States (USA), contamination of egg contents with S. Enteritidis is reported as the

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leading cause of human salmonellosis (Gast et al., 2024).

S. Enteritidis can contaminate eggs through two routes: external and internal. The horizontal route occurs when the bacterium is excreted in fecal matter, contaminating the eggshell during oviposition, then, it may penetrate the eggshell after laying and contaminate the contents, the vertical route occurs through colonization of the reproductive organs, leading to direct contamination of the egg components (yolk, albumin, testaceous membranes, vitellin, and eggshell) during synthesis in the reproductive tract. The Enteritidis serovar has a tropism for these sites after colonizing the intestine and disseminating systemically, where it can establish itself and be intermittently excreted in both eggs and feces (Gantois et al., 2009; Liu et al., 2023).

In 2004 in Mexico, the S. Enteritidis presence was reported in hens (Gallus gallus) for parents, breeders, incubators, commercial laying birds, eggs, and broilers (Mancera et al., 2004). Globally, the estimated prevalence of S. Enteritidis infection in laying hens is 40.2 % (Castro-Vargas et al., 2020) with approximately 1 in every 20,000 eggs contaminated (Gast et al., 2024). In the USA, the prevalence is estimated at 0.005 %, with a production of 90 billion eggs, the risk translates into an estimated 4.5 million contaminated eggs (Howard et al., 2012), in Europe the prevalence is 0.37 %, while in China, it ranges between 0.5 and 5.6 % (Solis et al., 2023). Xu et al. (2022) report that half of all salmonellosis cases in the USA are linked to the consumption of eggs or their raw byproducts, with an estimated economic burden of $3.7 billion per million cases. In Mexico, there are few reports on Salmonella spp. in hens; SENASICA (2021) reported losses of $27,614.58 pesos for the slaughter and death of 500 birds in Hidalgo in 2020, which tested positive for S. Gallinarum (fowl typhoid). Regarding cases of paratyphoid fever and other salmonellosis, Contreras et al. (2019) reported 104,471 cases in Mexico, while SENASICA, (2021) reported 42,000 cases of non-typhoid salmonellosis in humans.

Consumers play a fundamental role in salmonellosis prevention, particularly in the preparation and handling of egg containing foods at home. Several factors can lead to salmonellosis, for example, natural contamination of eggs, lack of consumer knowledge regarding food safety, cross-contamination, and the consumption of raw or undercooked eggs, the latter being considered the factor with the greatest impact (Poudel & Adhikari, 2024; Solis et al., 2023). The presence of S. Enteritidis in poultry farms depends on several factors among which are listed the type of housing, age of hens, farm population, production system, cleaning and disinfection strategies, presence of rodents and arthropods, and time of the year (Rodriguez et al., 2015). Regarding the type of housing and Salmonella contamination in eggs, Wall et al. (2008) reported higher shell contamination in enriched cages equipped with nest, perches and litter boxes compared to conventional cages, in the case of cage free housing, Pacholewicz et al. (2023) report lower percentage of Salmonella contamination compared to farms with cage housing. In Europe, the use of battery-type cages for housing hens has been banned since 2012. These were replaced by enriched systems that included nests, litter boxes, perches, and more space. However, cage free systems, whether floor based or free range, are the most widely accepted as they allow hens to express natural behaviors, improve animal welfare indicators, and reduce the incidence of Salmonella contamination in eggs (Solis et al., 2023; EFSA, 2017). Heat stress in eggproducing hens is another important factor affecting poultry production, causing inflammation

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in the gastrointestinal tract and influencing the composition and population of the microbiota that protects the intestine from the pathogen colonization, increasing the virulence and pathogenicity of Salmonella and facilitating the colonization of intestinal tissues and its systemic dissemination, and increasing bacterial excretion, thereby raising the risk of feed contamination (Lara & Rostagno, 2013).

In poultry production, hygiene and disinfection, immunization, use of enzymes, bacteriophages, probiotics, prebiotics, and antibiotics are employed to reduce the incidence of infections in poultry and, consequently, the frequency of salmonellosis in humans (Van immerseel et al., 2005; Rifat et al., 2022; Gast et al., 2024). However, this is not enough to eliminate the microorganisms from farms and hens, and the interaction between bacteria and antibiotics promotes resistance (Gast et al., 2015). In this regard, Borges et al. (2019) analyzed the antibiotic susceptibility of 163 Salmonella strains isolated from poultry samples, only 3 % (5/163) showed sensitivity to all antibiotics (amoxicillin, ceftiofur, chloramphenicol, gentamicin, and sulfamethoxazole/trimethoprim) and 30 showed multidrug resistance (resistance to three or more antibiotic classes), 9 of these were identified as S. Enteritidis. For this reason, this work aimed to determine the presence of antibiotic-resistant strains of Salmonella Enteritidis in the egg contents of hens housed in conventional cages in technified farms in Sinaloa, Mexico.

Material and methods

Study environment

The study was a cross-sectional, descriptive, and convenience study. The study area (Figure 1) covered the municipalities of the northern region of Sinaloa (municipalities of Ahome and El Fuerte), the central region (Culiacan municipality), and the southern region (municipalities of El Rosario and Mazatlan). In the northern region, 76 % of the egg producing hen farms registered in the state are located, accounting for 82.32 % of total egg production. The central and southern areas contain 9 % and 15 % of the farms, producing 4.99 % and 12.69 % of the egg output, respectively. The commercial farms where the samples were obtained use conventional cages (both battery and pyramidal) in natural environments, with manual egg collection. The hen populations are mainly of the Lohmann line, aged between 50 and 86 weeks. These conditions are similar to those on most other farms in the area.

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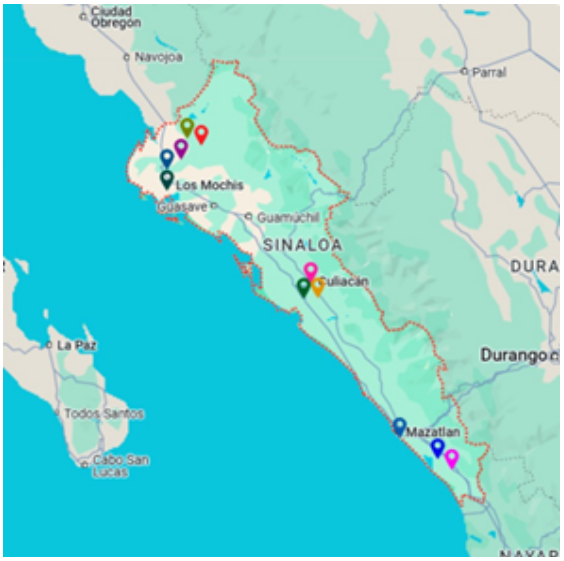

Figure 1. Map of the distribution of sampled farms in Sinaloa state, Mexico.

Sample collection

Egg collection and microbiological analyses were performed between November 2018 and April 2019. During the sampling period, nine companies with 28 farms were registered with the Poultry Farmers Association in Sinaloa. Of these, 2 farms were located in the central region, 2 in the south, and 24 in the north. The number of birds on these farms ranged from 35,000 on the smallest farm to 490,000 on the largest. All farms used conventional cages with natural environments, with a total population of 3,571,521 hens in production.

A convenience sampling method was used for the analysis, collecting 320 eggs from 11 farms. In the northern region, 150 eggs were collected from five farms, with hen populations of 92,750, 120,000, 180,000, 162,856, and 215,086 birds, respectively. In the central region, 90 eggs were collected from three farms, with populations of 40,000, 80,000, and 90,000 hens. In the southern region, 80 eggs were collected from three farms, with populations of 470,000, 490,000, and 135,000 hens, respectively. Due to outbreaks of highly pathogenic avian influenza in Mexico during the sampling period (SENASICA, 2024), it was impossible to access the farms directly. Therefore, the farm managers were asked to provide an egg box with 30 clean eggs from the daily production, which were destined for public sale (storage area before distribution). Bacteriological analysis began within 24 h of the arrival of samples at the laboratory.

Isolation of *Salmonella spp.*

Isolation and molecular identification were conducted at the Bacteriology and Mycology Laboratory of the Faculty of Veterinary Medicine and Animal Husbandry of the Universidad

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The eggshell was first cleaned with a plastic fiber and liquid detergent (Extran® MA 02 liquid, neutral) for the *Salmonella* Enteritidis isolation. The egg surface was gently scrubbed without fracturing it and then rinsed with running water. The eggs were subsequently immersed in a 200 ppm sodium hypochlorite solution for 3 min, and dried on sterile paper. Once disinfected and dried, the contents were poured into a sterile beaker, with care taken to discard broken eggs or those where eggshell fragments came into contact with the yolk or albumin. The contents were then homogenized with a sterile glass rod, and any samples contaminated by eggshell fragments were discarded. Pre-enrichment was performed by inoculating 12.5 mL of the homogenized egg contents (yolk and albumin) into 112.5 mL of buffered peptone water (1:9 ratio) and incubating at 37 ± 1 °C for 24 h. Selective enrichment was then carried out by transferring 1 mL of the pre-enrichment into 9 mL of Rappaport-Vassiliadis broth and incubating at 42 ± 1 °C for 24 h. Finally, isolation was done by streaking samples onto Hektoen enteric agar and xylose lysine deoxycholate (XLD) agar, followed by incubation at 35 ± 1 °C for 24 h (NOM-210.SSA1-2014; FDA, 2007).

Biochemical identification of *Salmonella spp.*

For metabolic characterization, typical *Salmonella* spp. colonies were inoculated onto triple sugar iron agar (TSI), lysine iron agar (LIA), Simmons citrate agar, indole sulfide motility (SIM) agar, nutrient gelatin, and urease broth. These cultures were incubated at 37 ± 1 °C for 24 h to characterize the metabolism of the suspected isolates (Moosavy *et al.,* 2015; NOM-210-SSA1-2014; Cepeda-Quintero *et al.,* 2022).

Molecular identification of strains of *Salmonella* Enteritidis

DNA extraction from the isolated bacteria was performed using the EZ-10 Spin Column Genomic DNA Minipreps kit for bacteriological cultures (BIO BASIC®). Identification was carried out by endpoint PCR with two pairs of oligonucleotides: one to amplify the *invA* gene for identification of the *Salmonella* genus, and another to identify the Enteritidis serotype using the *prot6E* gene (Afshari *et al.,* 2018). PCR was performed in a ThermoMixer C Eppendorf, the reaction volume was 10 µL: 5 µL of master mix, 1 µL of working solution of each oligonucleotide (10 µM), 1 µL of DNA, and 2 µL of sterile nano pure water. Cycling conditions included an initial denaturation step at 95 °C for 5 min, followed by 34 cycles of 95 °C, 60 °C, and 72 °C for 1 min each, with a final extension step at 72 °C for 5 min. DNA from *Salmonella* Enteritidis ATCC (13076) and *Escherichia coli* ATCC (25922) were used as positive and negative controls, respectively. The PCR product visualization was done by staining the amplified fragments in 2 % agarose gel using 1 µL of Biotum fluorescent dye (GelRed®) (Maurischat *et al.,* 2015).

Phenotyping of antibacterial resistance in *Salmonella* Enteritidis strains

Antibiograms were performed using the disc diffusion method (Kirby-Bauer) with multidiscs for Gram-negative bacteria (PT-35 multibac combined I.D.), which included chloramphenicol (CL), cefotaxime (CFX), ampicillin (AM), sulfamethoxazole/trimethoprim (SXT), ciprofloxacin (CPF), and nitrofurantoin (NF). The bacterial strain was inoculated in trypticase soy broth and incubated

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until it reached a concentration of 0.5 McFarland (1.5 x 10^8 CFU). The inoculum was then spread by continuous cross streaking on Mueller-Hinton agar, multidiscs were placed on the surface, and the plates were incubated at 37 °C for 24 h, once the established time had elapsed, the diameter of the growth inhibition halos was measured in mm for each antibiotic. Results were interpreted according to the criteria established by the Clinical and Laboratory Standards Institute (CLSI, 2021) and Diagnostic Research Laboratory (ID, 2020). All tests were performed in duplicate (Rivera *et al.,* 2013).

Statistical analysis

The isolation and antibiotic susceptibility test results were presented in a frequency table. A 95 % confidence interval (C.I.) was calculated using the binom.test function from the stats package in R 4.0.2 (R Core Team, 2020).

Results and discussion

Isolation of *Salmonella* spp in egg contents

The two colonies of *Salmonella* spp. (strain 1 and strain 16) isolated from chicken egg samples were biochemically identified through various tests. Their ability to ferment glucose and produce hydrogen sulfide was evaluated using triple sugar iron agar (TSI). In lysine iron agar (LIA), the presence of the enzyme decarboxylase, which metabolizes lysine, was tested. In Simmons citrate, the use of sodium citrate as the only carbon source was tested; in SIM, the motility of the isolates, and their capacity to produce H_2S and indole production were tested. The presence of proteolytic enzymes capable of hydrolyzing gelatin was tested in nutrient gelatin, and the capacity to degrade urea was identified with the urease test (Table 1).

Table 1. Biochemical Testing of *Salmonella* spp. Strains Isolated from Chicken Egg Yolk and Albumin.

Strain identification	TSI	LIA	Simmons citrate	SIM	Nutritious gelatin	Urea broth
Strain 1	+	+	+	+	-	-
Strain 16	+	+	+	+	-	-

TSI; triple sugar iron agar, LIA; lysine iron agar, SIM; sulfide indole motility, (+) positive for biochemical characteristics of *Salmonella* spp., (-) negative for biochemical characteristics of *Salmonella* spp.

Salmonella spp. were detected in 0.625 % [(95 % CI 0.076, 2.239) (2/320)] of the egg content samples tested, the strains were isolated from two different farms in the northern part of the state (Table 2).

Molecular identification through PCR identified the two isolates as *Salmonella* Enteritidis (Table 2), confirming that Enteriditis serovar is also present in eggs produced on poultry farms in Sinaloa and thus threatening the health of egg consumers.

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Efforts to reduce the prevalence of *Salmonella* Enteritidis in breeding and laying hen farms have led to the implementation of standards designed to decrease the number of positive farms for this and other serovars. In Mexico, NOM-159-SSA1-2016 mandates reporting the presence or absence of *Salmonella* in shell eggs and liquid eggs, while NOM-114-SSA1-1994 regulates the determination of *Salmonella* in food. In the European Union (EU), the European Food Safety Authority (EFSA) was established a reduction in contamination levels in egg producing hen farms to 2 % and breeder farms to 1 % (EFSA, 2019). Regulation No. 517/2011 and 2160/2003 of the Official Journal of the European Union requires reducing prevalence by 10-40 % compared to the previous year. Eggs from *Salmonella* positive flocks may only be used for human consumption if they undergo processes that destroy *Salmonella* serovars, such as pasteurization (Regulation No. 2160/2003).

Table 2. Isolation and molecular identification of *Salmonella* spp. strains isolated from chicken egg yolk and albumin.

Sinaloa State Area	Egg samples tested	Samples positive for *Salmonella* spp; n (%)	Positive samples for *S.* Enteritidis; n (%)
	150	2 (1.33)	2 (1.33)
North		CI95 % 0.162, 4.733	CI95 % 0.162, 4.733
Center	90	0	0
South	80	0	0
	320	2 (0.625)	2 (0.625)
Total		CI95 % 0.076, 2.239	CI95 % 0.076, 2.239

CI; Confidence Interval.

The determinations of S. Enteritidis presence as a biological agent causing contamination of the egg content of hens carried out in this work coincide with those reported in other countries. In Egypt, Abou et al. (2021) isolated S. Enteritidis in 3 % (5/165) of the eggs evaluated. Li et al. (2020) reported 0.3 % (2/666) of egg content samples testing positive for S. Enteritidis in China. In India, Shivaning Karabasanavar et al. (2020) detected S. Enteritidis strains in 2.1 % (4/186) of the samples analyzed. Similarly, Betancor et al. (2010) reported 1.2 % (8/620) of egg content samples contaminated with S. Enteritidis in Uruguay. In Mexico, Guzmán-Gómez et al. (2013) found Salmonella spp. contamination in 3 % (3/100) of egg yolk samples, and Mancera et al. (2005) confirmed the presence of S. Enteritidis in 0.25 % of egg samples marketed in supermarkets in Mexico City. Although the prevalence of S. Enteritidis is low in all these studies, the bacterium remains pathogenic, as infections can lead to salmonellosis outbreaks globally. For instance, in 1998, a hospital in Mexico City reported a salmonellosis outbreak that affected 155 health workers, traced back to the consumption of undercooked food containing meat, potatoes, and eggs contaminated with S. Enteritidis (Chávez et al., 2001).

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Antibiotic susceptibility testing

The two isolates of *Salmonella* Enteritidis demonstrated resistance to two classes of antibiotics: beta-lactams (ampicillin and cefotaxime) and quinolones (ciprofloxacin), which did not inhibit the growth of either strain. The strain identified as egg 16 also exhibited resistance to nitrofurantoin, a member of the nitrofuran class, thus categorizing it as multidrug-resistant (Table 3). Castro-Vargas *et al.* (2020) report that, globally, antibiotic resistance in *Salmonella* spp. is higher for ampicillin and nalidixic acid compared to other antibiotics. The presence of *S.* Enteritidis strains resistant to antimicrobials in egg contents poses a risk to consumers and compromises food safety. Infections caused by these antibiotic-resistant microorganisms represent a significant public health threat. It is crucial to implement actions to identifying and halting the spread of antimicrobial resistance. The strains isolated in this research exhibit phenotypic resistance to fluoroquinolones (ciprofloxacin), among other antibiotics. The WHO has issued an alert regarding the high morbidity and mortality associated with this bacterial genus due to reported drug resistance (WHO, 2024). This indicates that the strains have been exposed to antibiotics and have either acquired or inherited resistance genes that enable them to survive against these drugs. If current practices in the food industry continue, the number of infections caused by antibiotic-resistant organisms will likely increase, thereby reducing the availability of effective antibiotic treatments.

Table 3. Results of antibiotic susceptibility tests on *Salmonella* Enteritidis strains isolated from the egg contents of hens housed in cages in intensive production.

Strain identification	Area of origin	AM	CFX	CPF	CL	NF	STX
		10 µg	30 µg	5 µg	30 µg	300 µg	25 µg
	North	R (16mm)	R (25mm)	R	S	S	S
Egg strain 1				(26mm)	(21mm)	(18mm)	(20mm)
	North	R (16mm)	R (24mm)	R	S	R	S
Egg strain 16				(26mm)	(22mm)	(11mm)	(20mm)

AM: Ampicillin, CFX; Cefotaxime, CPF; Ciprofloxacin, CL; Chloramphenicol, NF; Nitrofurantoin, STX; Sulfamethoxazole; R; Resistant, S; Sensitive. interpretation of inhibition zones according to CLSI, 2021.

Abou *et al.* (2021) reported that *S.* Enteritidis strains in Egypt exhibited resistance to ampicillin (100 %) and high levels of resistance to amoxicillin (60 %), nitrofurans (80 %), and tetracyclines (88 %). In China, Li *et al.* (2020) detected resistance to ampicillin in 7 isolates (53.8 %) of *S. Enteritidis*, as well as one isolate resistant to chloramphenicol and another to sulfamethoxazole. Similarly, Shivaning Karabasanavar *et al*. (2020) in India found that 72.7% of isolates displayed multidrug resistance, including 44.5 % resistant to ampicillin and tetracycline, 40.9 % to ampicillin/sulbactam, and 72.2 % to nalidixic acid. Nabil & Yonis (2019) in Egypt reported that 94.1 % of *Salmonella* spp. strains were resistant to ampicillin and sulbactam, 70.6 % to tetracyclines, and 47.1 % to sulfamethoxazole/trimethoprim. Thung *et al. (*2016) also identified resistance to ampicillin. Campioni *et al.* (2012) determined the resistance profile of 128 *S. Enteritidis* strains

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isolated from clinical and food samples in Brazil, finding that 28.1 % were resistant to nalidixic acid and 0.8 % to sulfamethoxazole/trimethoprim. This reported antibiotic resistance highlights the need for stringent safety measures in laying hen farms, as zoonotic diseases originating from these sources can pose severe public health risks. The WHO emphasizes that bacterial resistance can lead to millions of deaths worldwide.

Conclusions

This is the first report of the presence of *Salmonella Enteritidis* in eggs from hens housed in conventional cages in technified farms located in the state of Sinaloa. Two strains (0.625 %) were isolated from egg yolk and albumin; both exhibited phenotypic resistance to two classes of antibiotics (2:2), while one strain (egg 16) showed resistance to three classes, categorizing it as a multidrug-resistant strain (1:2).

The presence and dissemination of this zoonosis through egg production in Sinaloa pose significant health risks to consumers. Furthermore, antibiotic resistance could undermine the effectiveness of medical treatments, thereby exacerbating these risks. It is crucial to determine the level of *Salmonella Enteritidis* contamination in laying hens in Sinaloa and to characterize the antibiotic resistance profiles. This knowledge is essential for developing strategies to reduce the presence of *Salmonella* on farms and the incidence of human salmonellosis.

Author contribution

Conceptualization of the state of the art of the subject of study: MACC, JJPL, CBCT. Methodology development: MACC, IEV, HCQ, JMVU, EPF, NCC.

Data management and analysis of results: MACC, JJPL, IEV, SMGC.

Manuscript writing and editing: MACC, JJPL, IEV, EPF, SMGC.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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