

# Antimicrobial Resistance in *Salmonella* spp. isolated from cattle gallbladder slaughtered in the south of the state of Rio de Janeiro

## Resistência a antimicrobianos em *Salmonella* spp. Isolada de vesículas biliares de bovinos abatidos no sul do estado do Rio de Janeiro

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### Abstract

*Salmonella* infections are an important cause of mortality and morbidity in cattle, and subclinically infected animals are frequently found. The microorganism may exhibit multiple antibiotic resistance and may be able to survive in harsh environments such as the gallbladder. *Salmonella* Multiple Antibiotic Resistance (MAR) was detected in bile and gallbladder epithelium from cattle slaughtered in a plant in southern state of Rio de Janeiro, Brazil, under sanitary conditions. The frequency of resistance to each drug was not significantly different between bile and epithelium, except in the case of cefotaxime, which showed increased resistance in the bile. Aztreonam was the most effective antibiotic for inhibiting strain growth. Since the results showed *Salmonella* spp. strains resistant to twelve antimicrobials, it denotes an alarming risk, therefore in cases of invasive infections in both human and animals there will be reduction of therapeutic options against the pathogen.

Keywords: *Salmonella* spp., gallbladder, cattle, antimicrobials, antibiotic resistance.

### Resumo

Infecções causadas por *Salmonella* spp. são uma importante causa de mortalidade e morbidade em bovinos, e animais subclínicamente infectados são frequentemente encontrados. *Salmonella* spp. pode expressar múltipla resistência a antibióticos e ser capaz de sobreviver em ambientes inóspitos, como a vesícula biliar. *Salmonella* spp. com múltipla resistência a antibióticos foi detectada na bile e epitélio de vesículas biliares de bovinos abatidos em matadouro-frigorífico no sul do estado do Rio de Janeiro, Brasil, sob Inspeção Sanitária. A frequência de resistência a cada droga não foi significativamente diferente entre bile e epitélio, exceto no caso de cefotaxima, que mostrou grande resistência na bile. Aztreonam foi o antimicrobiano mais eficaz para inibir o crescimento da estirpe. Como na pesquisa foram identificadas salmonelas resistentes a 12 antimicrobianos testados, este patógeno torna-se alarmante, pois em casos de infecção invasiva em animais e humanos haverá redução de opção terapêutica.

Palavras-chave: *Salmonella* spp., vesícula biliar, bovino, antimicrobianos, resistência a antibióticos.

### Introduction

*Salmonella* infections are an important cause of mortality and morbidity in cattle, and subclinically infected animals are frequently found. Cattle may constitute an important reservoir for human infections (Wray & Davies, 2000). This genus exhibits resistance to many antibiotics and could become a problem in both human and veterinary medicine (Zhao et al., 2007). In cattle populations where antimicrobials are used, *Salmonella* species are under high selection pressure, and generic strains begin to present Multiple Antibiotic Resistance (MAR) (Defrancesco et al., 2004). According to data from SINDAN (2009), antibiotics are used extensively in livestock in Brazil; the amount spent on bovine species in 2009 was the equivalent of approximately one billion US dollars.

To survive in their host organism and spread throughout the food chain, *Salmonella* develop mechanisms that enable them to form permanent colonies and biofilms in harsh environments, triggering the establishment of chronic, asymptomatic carriers (Prieto et al., 2004; Dutta et al., 2000; Prouty et al., 2002; Steenackers et al., 2011). The gallbladder presents an example of adaptation by the microorganism in that *Salmonella* are highly resistant to bile (Gunn, 2000; Prouty et al., 2002; Van Velkinburgh and Gunn, 1999). To withstand the adverse conditions found in the gallbladder, including variable pH, low oxygen and nutrient limitation (Begley et al., 2005), and still be resistant to many antimicrobial agents, extensive gene regulation is required. Bile induces drug resistance in the organism and the expression of genes required for pathogenesis (Prouty et al., 2004a; Prouty et al., 2004b).

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Outbreaks of salmonellosis are common all over the country and often come from animal products. In addition, the observation that the gallbladder supports a high number of *Salmonella* strains suggests the possibility that cattle might be a persistent carrier of pathogens emerging as a risk to public health. The objective of the present study was to detect the presence of *Salmonella* strains in bile and gallbladder epithelium from cattle slaughtered in a plant under sanitary conditions and to evaluate the frequency of antimicrobial resistance in these strains.

## Material and methods

### Samples

Intact gallbladders were collected from 30 randomly selected healthy cattle, judged after *ante-mortem* and *post-mortem* inspection, in a slaughtering plant under sanitary conditions in the southern state of Rio de Janeiro, Brazil. Immediately after collection, the material was transported to the laboratory under refrigeration. Microbiological analysis was performed on the same day.

### Microbial Analysis and Phenotypic Identification

The gallbladder was opened in aseptic conditions, bile was collected and the biliary epithelium was sectioned with the aid of sterilized instruments. Bile and gallbladder epithelium were analyzed separately for *Salmonella* spp., as described by Pignato et al. (1995). Twenty-five grams of bladder epithelium and 25 ml of liquid bile were added to 225 ml of preenrichment Salmosyst Base broth (Merck) in a sterile bag. The sterile bags were homogenized in a stomacher for 3 minutes and incubated for 6 hours at 37°C. For the selective Salmosyst enrichment, 10 ml of preenrichment broth base was supplemented with one selective supplement tablet (Merck) and incubated for 18 hours at 37°C. Colony isolation was carried out in Rambach Agar (Merck), and typical colonies, after confirmation by Gram morphology, were subjected to biochemical identification using tests to verify the presence of cytochrome oxidase (Newprov®, Brazil), production of urease in urea broth (Himedia), fermentation of glucose, sucrose and lactose in Triple Sugar Iron (TSI) medium (Himedia), lysine decarboxylation in Lysine Iron Agar (LIA) medium (Himedia); production of H<sub>2</sub>S in Sulfide Indole Motility (SIM) medium (Himedia) and Voges-Proskauer reaction in Methyl Red-Voges Proskauer (VM-VP) broth (BioChemika – Sigma-Aldrich). In addition, serological tests were performed using somatic polyvalent *Salmonella* “Vi” and “H” antisera (Probac, Brazil).

### Antimicrobial Susceptibility Test

Following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2005), the antimicrobials used in the test were as follows: amikacin, tetracycline, cephalothin, cefotaxime, ceftazidime, aztreonam, cefoxitin, ceftriaxone and chloramphenicol (30 µg/disc) as well as sulphazotrin (25 µg/disc) and gentamycin and ampicillin (10 µg/disc). Strains of

*Salmonella*, grown on Case agar (Merck) for 24 hours at 37°C, were inoculated in 4 ml of sterile distilled water to achieve turbidity standard n<sup>o</sup>1 of the McFarland scale, corresponding to 10<sup>8-9</sup> CFU/ml. A swab dipped in the solution was used to spread the inoculum across the surface of Muller Hinton agar, and disks containing the antibiotics (DME Polissensidisc® 4x6-Specialized Diagnostic Microbiology, São Paulo, Brazil) were applied to the plate. The resistance of the strains was assessed by measuring the inhibition of bacterial growth after incubation for 24 h at 37°C. *Escherichia coli* ATCC 25922 was used for quality control testing.

### Statistical Analysis

Fisher's exact test was performed to test the correlation between the frequencies of antimicrobial resistance in the bile and gallbladder epithelium. A *P* value less than 0.05 was considered significant. The software used for statistical analysis was Biostat 2.0.

## Results and discussion

In this analysis of 30 gallbladders, *Salmonella* spp. were identified by phenotypic characteristics in 23 samples. The microorganism was found more frequently in the gallbladder epithelium, with 18 samples testing positive. *Salmonella* was found in 16 bile samples, and in 11 samples, *Salmonella* was found in both the bile and the gallbladder epithelium (Table 1). The growth of this microorganism in the gallbladder is due to the development of several molecular mechanisms, including the presence of a lipopolysaccharide in the external membrane that acts as a barrier against bile, as described by Picken and Beachmam (1977), and the action of a limited number of genes, such as *acrAB* (Nikaido et al., 1998; Ma et al., 1994), *phoP* (Van Velkinburgh and Gunn, 1999), *tolR* (Prouty et al., 2002) and *wec* (Ramos-Morales et al., 2003). In antimicrobial susceptibility testing, strains of *Salmonella*

**Table 1:** *Salmonella* spp isolated from the liquid and epithelium of gallbladders from 30 cattle slaughtered

Gallbladders	<i>Salmonella</i> spp isolated of gallbladders
Bile (n=16)	2, 3, 4, 5, 8, 10, 11, 13, 15, 16, 17, 21, 25, 27, 28, 30
Epithelium (n=18)	4, 5, 6, 7, 8, 9, 10, 13, 16, 17, 19, 20, 22, 25, 27, 28, 29, 30

from both bile and gallbladder epithelium showed resistance to the antimicrobials cephalothin, sulfazothrim and ampicillin (Table 2). The high frequency of resistance to cephalothin was expected because it is a first-generation cephalosporin with weaker activity against Gram-negative bacteria, as observed by Spinosa et al. (2002). According to Davis et al. (2007), the AmpC beta-lactamase gene (*cmx-2*) is responsible for encoding resistance to cephalosporin spectrum in *Salmonella* spp. Zhao et al. (2007), analyzed 129 cattle and found that 66% of *Salmonella* strains were resistant to ampicillin. Davis et al. (2007) reported that between 2001 and 2004, 79.6% of *Salmonella* Dublin isolates from cattle were resistant to ampicillin and 32.7% to trimethoprim-sulfa.

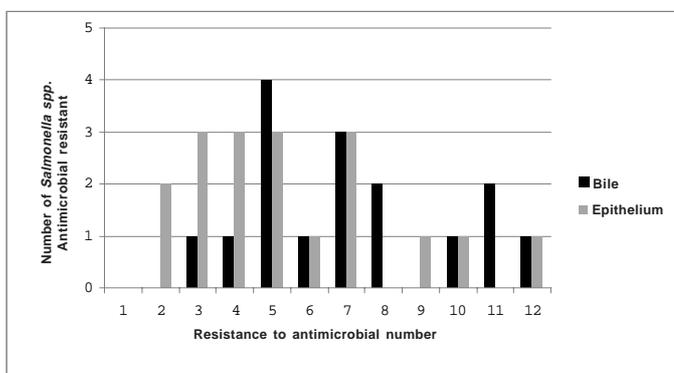
**Table 2:** Percentage of *Salmonella* strains resistant to antimicrobials isolated from the liquid and epithelium of the cattle gallbladder slaughtered

Antimicrobials	Bile (n=16)	Epithelium (n=18)	P-value <sup>a</sup>
Amikacin	37.50	33.33	1.00
Ampicillin	81.25	66.66	0.68
Cephalothin	93.75	94.44	1.00
Cefotaxime	62.50	16.66	0.01
Ceftadizime	37.50	16.66	0.68
Sulfazothrim	93.75	72.22	0.18
Aztreonam	25.00	16.66	0.68
Cefoxitin	68.75	61.11	0.72
Ceftriaxone	37.50	33.33	1.00
Chloramphenicol	50.00	33.33	0.48
Gentamicin	62.50	55.55	0.73
Tetracycline	68.75	50.00	0.31

<sup>a</sup> Fisher's exact test

Although resistance to chloramphenicol was only present in about 50% of *Salmonella* strains present in the bile, Prouty et al. (2004a) reported that pre-exposure to bile at sublethal concentrations confers resistance to chloramphenicol by activating the bile-induced operon *marAB*.

In this study, 6.25% of the strains present in bile and 5.55% of those in the gallbladder epithelium were resistant to 12 antimicrobials (Figure 1). The indiscriminate use of antimicrobials in livestock directly contributes to the development of *Salmonella* MAR. This fact has alarming consequences for public health because it reduces the number of therapy options in cases of invasive infection in animals and humans (Zhao et al., 2007).

**Figure 1:** Resistance profile of *Salmonella* strains isolated from bile and gallbladder epithelium of cattle slaughtered

In gallbladder epithelium, the highest sensitivity (Table 2) occurred with the drugs cefotaxime, ceftadizime and aztreonam, and in bile, sensitivity was increased to aztreonam, which should therefore be considered the drug of choice for the control of *Salmonella* spp. in cattle. Our findings corroborate those of Chandra et al. (2005), who isolated

species of *Salmonella* in the gallbladder and mesenteric lymph nodes of goats and also found aztreonam to be effective against most serovars.

In this study, the frequencies of resistance to each drug in bile and gallbladder epithelium (Table 3) were not significantly different by Fisher's exact test ( $P < 0.05$ ). Therefore, resistance to certain drugs is similar in both environments, with the exception of cefotaxime, to which microorganisms in bile showed significantly higher resistance ( $P = 0.01$ ). When comparing the frequency of multidrug resistance of strains from bile and epithelium of the same organ (Table 3), Fisher's exact test did not show any statistical difference, although the average drug resistance in bile was higher than the average found in gallbladder epithelium. In biliary fluid, bacteria can sometimes achieve greater resistance to a drug after being in direct contact with bile and, in the epithelium, bacteria may survive by aggregating to form a protective layer. According to Prouty et al. (2004a), the presence of bile salts in these bacteria induces the expression of genes that activate the efflux pump to expel the bile and, consequently, the antimicrobial agents.

**Table 3:** Resistance expressed by *Salmonella* strains isolated in the liquid and epithelium from the same gallbladder to number of antimicrobials tested\*

Gallbladder with <i>Salmonella</i> isolated in bile and epithelium	Resistance of <i>Salmonella</i> spp. to number of antimicrobials		
	Bile	Epithelium	P-value <sup>a</sup>
4	12	9	0.21
5	7	10	0.37
8	7	2	0.08
10	5	2	0.37
13	10	7	0.37
16	6	7	1.00
17	4	4	1.33
25	5	5	1.31
27	5	4	1.00
28	5	6	1.00
30	8	5	0.41
Mean	6.72	5.54	---- <sup>b</sup>

\*12 antimicrobials tested

<sup>a</sup> Fisher's exact test.

<sup>b</sup> Value no calculated.

## Conclusion

The ability of *Salmonella* spp. to survive the harsh environment of the gallbladder and become resistant to antimicrobials highlights the need for special care to be taken during the raising and slaughter of cattle in order to prevent contamination.

The use of drugs in livestock should be restricted. At slaughter, extreme caution is required to avoid the contamination of carcasses, parts of carcasses, organs and viscera, equipment surfaces, instrumental work and handlers' hands with bile and gallbladder epithelium. Because gallstones have considerable market value as research samples, it is common to open the gallbladder at the slaughterhouse. As a result, bile is frequently spilled, often on the liver.

Restriction of antimicrobial use in livestock and greater care when handling the gallbladder would have a prophylactic effect on the dissemination of MAR strains of *Salmonella*. Without these measures, the horizontal transmission of strains

through the food chain will lead to serious consequences for public health by limiting the number of antibiotics that are effective against these microorganisms.

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