

CHARACTERIZATION OF BACTERIAL MICROBIOTA PRESENT IN BILE AND GALLBLADDER EPITHELIUM OF CATTLE*

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ABSTRACT

Pathogenic microorganisms can reside transiently or permanently in the gallbladder of cattle. Thus, during slaughter, more attention should be given to the gastrointestinal tract, especially to the accessory organ, the gallbladder. The aim of this study was to characterize the bacterial microbiota present in bile and gallbladder epithelium of cattle slaughtered in a slaughtering plant under sanitary conditions. Thirty intact gallbladders were collected and the presence of Total Aerobic Mesophilic Bacteria (TAMB), *Staphylococcus* spp., *Enterobacteriaceae*, *Escherichia coli*, *Enterococcus* spp. and *Salmonella* spp. in bile and epithelium were evaluated. The frequency of isolation of the microorganism mentioned above were, respectively: 23.02%, 14.39%, 13.67%, 24.46%, 0% and 24.46%. The frequency of microorganisms isolation the gallbladder epithelium was 64.03% and in the bile was 35.97% but no statistical difference were found. Nevertheless, a significant difference between the population averages can be observed. *Staphylococcus* strains from bile and gallbladder epithelium showed sensitivity to penicillin G, ceftriaxone, chloramphenicol and gentamicin. The high frequency of microorganisms in the gallbladder brings us to the possible fact that cattle be a persistent carrier of

pathogens of great importance to public health.

Keywords: Cattle gallbladder. Pathogenic microorganisms. Persistent carrier. Public health.

INTRODUCTION

Bile is a digestive secretion that plays a major role in the dispersion and absorption of fats. The composition of bile is complex, but can be thought of as lipid rich and protein poor (GUNN, 2000). Although the bile acids possess potent antimicrobial activity, recent research has shown that the bacterium is capable of tolerating high levels of bile and biofilm formation by pathogenic genera (BEGLEY; KER; HILL, 2009; STEENACKERS et al., 2012).

The ability of an organism to tolerate the bile is complex and involves an extensive range of proteins, including those which are responsible for the cellular membrane structure or maintenance of intracellular homeostasis. Proteins and enzymes modify and transform bile salts and also play an important part regarding the resistance of microorganisms to the bile (BEGLEY; GAHAN; HILL, 2005) and their own adaptive mechanism to support the environment (RYCHLIK; BARROW, 2005).

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The gallbladder may be a site of persistence and reservoir of certain enteric food-borne pathogens resistant to many antimicrobial agents. *Salmonella* to form permanent colonies and biofilms in gallbladders, triggering the establishment of chronic, asymptomatic carriers (STEENACKERS et al., 2012). Bacteria in biofilms are generally well protected against environmental stresses, antibiotics, disinfectants and the host immune system (JENSEN et al., 2010) and as a consequence are extremely difficult to eradicate.

Previous studies (MCDONOUGH; SHIN; LEIN, 2000; STOFFREGEN; POHLENZ; NYSTROM, 2004; REINSTEIN et al., 2007; JEONG; KANG; HEINKE, 2007) reported isolation of *E. coli* O157:H7 and *Salmonella* in gallbladders of cattle and suggested that the organism can reside in the organ this animals. Then, during the slaughter, greater attention must be given to the gastrointestinal tract of cattle, especially for gallbladder, implicated as a site, source and reservoir for *Salmonella* spp. and *Escherichia coli* among other pathogens. The aim of this study was to characterize the bacterial microbiota present in bile and gallbladder epithelium of cattle slaughtered in a slaughtering plant under sanitary conditions and evaluated the frequency of antimicrobial resistance in strains of genus *Staphylococcus*.

MATERIAL AND METHODS

Intact gallbladders were collected from 30 randomly selected healthy cattle, with *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.

The gallbladder was opened in aseptic conditions, bile was collected and the biliary epithelium was sectioned and analyzed separately for Total Aerobic Mesophilic Bacteria (TAMB), *Enterobacteriaceae*, *Staphylococcus*

aureus, *Escherichia coli*, *Enterococcus* spp. and *Salmonella* spp. To the first five analyzes cited, a total of 10 mL of bile and 10 g of gallbladder epithelium were aseptically transferred to 90 mL of 1% peptone water (Difco Lab., Detroit, Mich., U.S.A.) and homogenized for 2 min in a stomacher (Mayo Homogenius HG 400, São Paulo, Brazil) and serial decimal dilutions until 10^{-5} were realized for both samples.

The TAMB and total *Enterobacteriaceae* count were realized as described by Brasil (2003) and colony isolation was carried out in Plate Count Agar (PCA, Merck) and Red Bile Glucose (VRBG) Agar (Merck), respectively. Plates were incubated at 37°C for 48 hours. Gram staining, oxidase, nitrate reduction and oxidation and fermentation (O/F) of glucose were performed to strains differentiation on Enterobacteriaceae family. The Fluorocult LMX Broth (Merck) was used to detection of *E. coli*. Samples positives to Kovac's reagent were streaked on Eosin Methylene Blue (EMB) Agar (Merck). The API20E kit (BioMérieux) was used to complement the biochemical tests to *E. coli* and final identification was performed using the API LAB Plus software (BioMérieux).

The analytical procedures to *Staphylococcus aureus* isolation followed the technique described by Brasil (2003). Aliquots of 0.1 mL of different dilutions were streaked, in duplicates, on the Baird-Parker agar surface. Typical colonies were selected and Gram staining, catalase, coagulase, thermonuclease, DNase, oxidation/fermentation (O/F) of glucose and mannitol tests were used to characterize the genus: Enumeration of *Enterococcus* spp. was according to Merck (2000), the tubes with Chromocult broth (Merck) were inoculated and incubated by 48 hours at 45°C. Tubes positive for *Enterococcus* (bluish color) were streaked on Chromocult Agar (Merck) and incubated at 35°C for 48 h. Typical colonies were tested for Gram staining.

The genus *Salmonella* was detected as described by Pignato et al. (1995). Briefly, 25 mL of bile and 25 g of gallbladder epithelium were aseptically

transferred to 225 ml of preenrichment Salmosyst broth (Merck), homogenized in stomacher for 4 min and incubated at 37°C by 6 hours; 10 ml of preenrichment broth was supplemented with one selective supplement tablet (Merck) and incubated for 18 hours at 37°C. A loopful of broth was streaked on Rambach agar plates (Merck) and incubated at 37°C by 24 hours. Typical colonies on Rambach agar were selected and transferred to tubes containing Agar Triple Sugar Iron (TSI) (Himedia) and Lysine Iron Agar (LIA, Himedia), incubated at 37°C by 24 hours. Characteristic strains of *Salmonella* spp. were tested for differential staining of Gram, catalase and oxidase. To complement these biochemical tests was used API20E kit (BioMérieux) and the final identification was performed using the API LAB Plus software (BioMérieux).

Antimicrobial susceptibility test was performed by penicillin G (10 UI/disc), teicoplanin (30 µg/disc), vancomycin (30 µg/disc), chloramphenicol (30 µg/disc), erythromycin (15 µg/disc), gentamycin (10 µg/disc), clindamycin (2 µg/disc), tetracycline (30 µg/disc), aztreonam (30 µg/disc), ceftriaxone (30 µg/disc), ceftiofexim (30 µg/disc) and oxacillin (1 µg/disc) were used following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2011). *Staphylococcus* strains were grown on Caso Agar (Merck) for 24 hours at 37°C. The strains were inoculated in 4 ml of sterile distilled water to achieve the n° 0.5 McFarland turbidity standard (Probac, Brazil). A swab was used to spread the inoculum across the surface of Muller Hinton agar (Merck), and then antibiotic disks (DME Polissensidisc® 4x6-Specialized Diagnostic Microbiology, São Paulo, Brazil) were applied to the

plate. Strain resistance was assessed by measuring the zone of inhibition of bacterial growth after incubation for 24 h at 37°C. *Staphylococcus aureus* ATCC 25923 was used for quality control testing.

In the statistical analyses were performed the tests: Chi-Square and Student T to isolated microorganisms in the samples. Antimicrobial resistance to *Staphylococcus* strains in the bile and gallbladder epithelium was correlated by Fisher's exact test. The Biostat 2.0 software was used for all statistical analysis.

RESULTS AND DISCUSSION

The frequency of microorganisms can be observed in Table 1. Species of *Enterococcus* were not isolated in the samples of gallbladder and a higher percentage of *Salmonella* spp. and *E. coli* was found in both kinds of samples. The frequency of microorganisms in the gallbladder epithelium and the bile was not statistically different, corresponding to 64.03% and 35.97%, respectively. The frequency of microorganisms in the gallbladder was the same, this fact suggest that either in the bile as the gallbladder epithelium are a common environment for the microorganisms isolated. According to Hancke, Nusche and Marklein (1986), the isolation of bacteria in the bile normally coincides with the presence of bacteria in the epithelium, but the frequency of isolation of microorganism is greater in epithelium. This fact is probably because the microorganisms replicate within gallbladder epithelial cells, such as, *Salmonella* (Menendez et al., 2009; Steenackers et al. 2012).

Table 1 - Frequencies of microorganisms isolated from the bile and gallbladder epithelium of 30 cattle slaughtered.

Microorganism	Frequency of isolates			Percentage (%)		
	Bile	Epithelium	Total	Bile	Epithelium	Total
<i>Salmonella</i> spp.	16	18	34	11.51	12.95	24.46
TAMB	8	24	32	5.75	17.27	23.02
<i>E. coli</i>	13	21	34	9.35	15.11	24.46
<i>Enterobacteriaceae</i>	5	14	19	3.60	10.07	13.67
<i>Enterococcus</i> spp	0	0	0	0	0	0
<i>Staphylococcus</i> spp.	8	12	20	5.76	8.63	14.39
Total	50	89	139	35.97	64.03	100.00

Chi-square (p >0,05).

Salmonella resides in the gallbladder of cattle and goats and contributes to the persistent carrier status of these animals (WOLDEMARIAMA et al., 2005). Previous studies report the presence of *Salmonella* in gallbladders of ruminants (MCDONOUGH; SHIN; LEIN, 2000; CHANDRA; SINGH; SHANKAR, 2006; AKOACHERE et al., 2009). According to Van Velkinburgh and Gunn (1999), the *Salmonella* are highly bile resistant, with MBC (Minimal Bactericidal Concentration) between 30 and 60%. Thus, the responses of *Salmonella* to bile are important for virulence and also the establishment of chronic infections or carrier states in the host (SPECTOR; KENYON, 2012).

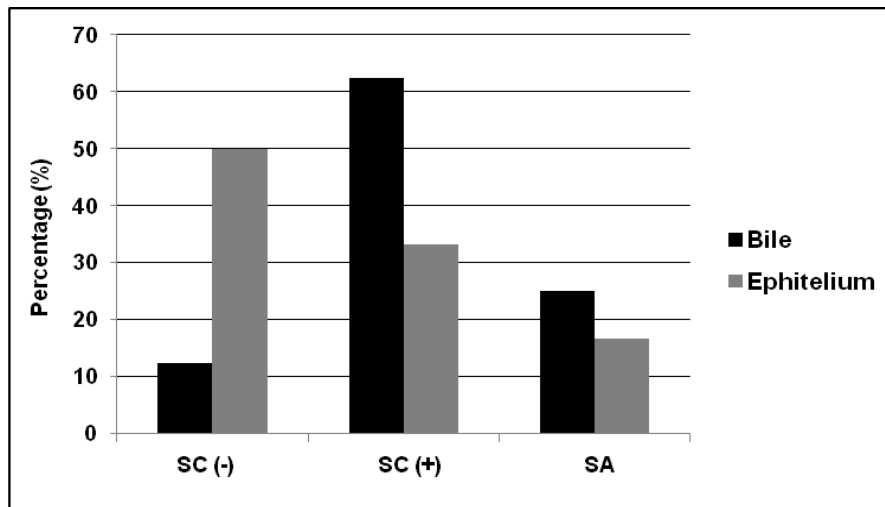
Another Gram negative bacteria, isolated with the same frequency as *Salmonella* spp was *E. coli*, although great part of it was adhered to the epithelium. The enterobacteria counting presented 13.67% of isolations in the samples, prevailing higher count in the epithelium (Table 1). Contrepois et al. (1986); Onyekaba and Njoku (1986); Stoffregen, Pohlenz and Nystrom (2004); Reinstein et al. (2007) and Jeong, Kang and Heinke (2007) detected *E. coli* in the gallbladders of cattle demonstrating that this organism can live permanently in the organ. Second Stoffregen, Pohlenz and Nystrom (2004), the gallbladder is a possible niche for *E. coli* in cattle, as it is for other enteric pathogens, notably *Salmonella* spp. The gallbladder may be a site of and a source of gastrointestinal Shiga Toxin-producing *E. coli* (STEC), which can contaminate beef products.

The TAMB frequency was of 23.02%, being in most parts, isolated from the epithelium (Table 1). The occurrence

of TAMB and other prokaryotes found in the gallbladder, call our attention to care and needs for changes in the current method of liver inspection as, according to the standardization of techniques for cattle slaughtering (BRASIL, 1971), in the *post-mortem* exam of cattle liver, the gallbladder is compressed, and incised as needed. 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 (DIAS et al., 2010). Therefore, evisceration should be done carefully and the bile duct should be tied before removing gallbladder to prevent cross-contamination with bile.

The frequency of *Staphylococcus* spp. was considered high in this research, of 14.39% where 8.63% were present in the epithelium of the gallbladder and the other corresponding 5.76% in the bile (Table 1). Biochemical tests allowed the identification of coagulase negative *Staphylococcus*, coagulase positive *Staphylococcus* and coagulase positive *Staphylococcus aureus* present in the epithelium and bile. In the bile the highest percentage of staphylococci found corresponded to coagulase positive *Staphylococcus* and in the epithelium was *Staphylococcus* coagulase negative (Figure 1). The detection of *S. aureus* in the gallbladder is more an alert to avoid cross-contamination by evisceration, especially on the liver. *Staphylococcus aureus* are capable of producing heat-stable toxins that are frequently responsible for food poisoning in humans (LOIR; BARON; GAUTIER, 2003).

Figure 1 -Frequency (%) of coagulase negative *Staphylococcus* (SC (-)), coagulase positive *Staphylococcus* (SC (+)) and *S. aureus* (SA) present in the bile and epithelium of gallbladders from 30 cattle slaughtered.



Gram positive bacteria seem to be more sensitive to the deleterious effects of bile than Gram negative bacteria and bile salts are often used in their selective enrichment (e.g. Mac Conkey agar, Salmonella-Shigella agar, violet red bile agar and bile esculin agar) (BRIDSON, 1995). However, in this study, there was the detection of microorganism Gram positive in bile. Begley, Gahan and Hill (2005) and Begley, Ker and Hill (2009) demonstrated that *Listeria monocytogenes* strain (Gram positive bacteria) is able to tolerate concentrations of bovine, porcine and human bile well in excess of those encountered *in vivo*. *Clostridium* spp. and *Clostridium perfringens* were isolated from human and animal bile suggesting an inherent bile tolerance in this pathogen (SAKAGUCHI; MURATA; KIMURA, 1983; CABLE; REHBUN, FORTIER, 1997). Ohtomo, Yoshida and San Clemente (1981) also demonstrated the capacity of growth of *S. aureus*, through encapsulation, in several concentrations of biliary acids. Liau and Hash (1977) affirmed that the taurine (a compound present in conjugated biliary acids) was detected in the capsular material produced by *S. aureus* strains, being one of the components of the Surface Polysaccharide of the Antigen (SPA).

The effect of bile on Gram positive enteric bacteria is not well understood, but some of the mechanisms found in Gram

negatives are likely to be found in Gram positive enteric bacteria as well. An observation made by several investigators is that bile tolerance is a strain-specific trait and tolerances of species cannot be generalized (BEGLEY; GAHAN; HILL, 2005). Thus, the result found in this study allows questioning of the theoretical basis that bile salts are inhibitors of Gram positive bacteria and used as a component in bacteriological media selective for Gram negative microorganisms.

Antimicrobial susceptibility test showed that the strains of *Staphylococcus* were sensitivity to penicillin G, ceftriaxone, chloramphenicol and gentamicin in both bile and gallbladder epithelium (Table 2). Moreover, strains of *Staphylococcus* were sensitive to teicoplanin and vancomycin in the gallbladder epithelium. The frequency of resistance in the bile and the epithelium was statistically significant to some antimicrobials. Staphylococcal strains present in the epithelium were more susceptible to erythromycin, teicoplanin, vancomycin and cefoxitin. This can be explained by a larger number of bacterial are aggregated in the epithelium. Due to high bacterial population and the low availability of nutrients, there is occurrence of a low metabolic rate of microbial cells as reported by Cloete (2003), which fits the gallbladder, and becomes an

interfering factor of antimicrobial resistance in the microbial population. **Table 2** - Percentage of *Staphylococcus* strains resistant to antimicrobials isolated from the bile and epithelium of the cattle gallbladder slaughtered.

Antimicrobials	Bile (8 strains)	Epithelium (12 strains)	P- value ^a
Clindamycin	0.00	16.66	0.49
Erythromycin	87.50	25.00	0.01
Oxacillin	0.00	8.30	1.00
Penicillin G	0.00	0.00	----*
Teicoplanin	37.50	0.00	0.04
Vancomycin	50.00	0.00	0.01
Aztreonam	87.50	91.66	1.00
Cefoxitin	87.50	33.33	0.02
Ceftriaxone	0.00	0.00	----*
Chloramphenicol	0.00	0.00	----*
Gentamicin	0.00	0.00	----*
Tetracycline	75.00	50.00	0.37

^a Fisher's exact test

* Value not calculated. Susceptible strains in both environments.

In this survey, one *Staphylococcus* strain present in bile and two *Staphylococcus* strains isolated in the gallbladder epithelium were resistant to seven and five antimicrobials tested, respectively. The ability of *Staphylococcus* 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.

In relation to the bacterial numbers, there were variations between the value found for the epithelium and the bile (Table 3), prevailing the highest average for the epithelium in the gallbladder. By the Student T test, there was significant difference between log₁₀ CFU/g and log₁₀ CFU/mL population averages in microorganisms present in both environments of the gallbladder.

Considering the results obtained in this study, it can be affirmed that the environment for the microorganism in the gallbladder is common. The bacteria can be isolated in the bile as well as in the epithelium, but in larger quantities in the epithelium. It can be suggested that the frequency and average counting of microorganisms in the gallbladder is due to its adherence to the epithelium, being its common habitat but due to contractile function of the gallbladder, occurs the detachment of the bacteria from the epithelium and consequent migration toward the bile. According Begley, Ker and Hill (2009) and Steenackers et al. (2012) intestinal pathogens is also able to adhere to and subsequently form bacterial communities, microcolonies and even mature biofilms on epithelial cells.

Table 3 - Average count log₁₀ (CFU/mL and CFU/g) of microorganisms present in bile and epithelium of the cattle gallbladders. *

Microorganisms	Bile (CFU/mL)	Epithelium (CFU/g)
TAMB	3.25 ^a	6.46 ^b
<i>E. coli</i>	3.08 ^a	5.38 ^b
<i>Enterobacteriaceae</i>	3.67 ^a	6.11 ^b
<i>Staphylococcus</i> spp.	3.72 ^a	5.76 ^b

* For each row, mean values with different letters are significant ($P < 0.05$) according to the Student T test.

CONCLUSION

Pathogenic microorganisms can reside transiently or permanently in the gallbladder of cattle. This organ can play an important role as a contamination source of intestinal pathogens. Therefore, to control the bacterial contamination to meat products in slaughterhouse plants must be carefully observed. Attention should be targeting not only the gastrointestinal tracts but also the accessory organ like the gallbladder. Moreover, gallbladder of cattle should be recognized to a reservoir of some pathogens multidrug resistant of great importance to public health.

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CARACTERIZAÇÃO DA MICROBIOTA BACTERIANA PRESENTE NA BILE E EPITÉLIO DA VESÍCULA BILIAR DE BOVINOS

RESUMO

Microrganismos patogênicos podem residir temporariamente ou permanentemente na vesícula biliar de bovinos. Assim, durante o abate, maior atenção deve ser dada ao trato gastrointestinal, especialmente para o órgão acessório, a vesícula biliar. O objetivo do estudo foi caracterizar a microbiota bacteriana presente na bile e epitélio de vesículas biliares de bovinos abatidos em matadouro frigorífico sob inspeção sanitária. Foram coletadas 30 vesículas biliares íntegras e pesquisadas na bile e epitélio do órgão a presença de Bactérias Aeróbias Mesófilas Totais (BAMT), *Staphylococcus* spp. e *Enterobacteriaceae*, *Escherichia coli*, *Enterococcus* spp. e *Salmonella* spp. A frequência de isolamento dos microrganismos supracitados foi de 23,02%, 14,39%, 13,67%, 24,46%, 0% e

24,46%, respectivamente. Em relação aos dois ambientes da vesícula, a frequência de isolamento dos microrganismos no epitélio foi de 64,03%, e na bile, 35,97%, não sendo diferente estatisticamente, mas com diferença significativa entre as médias populacionais. No teste de susceptibilidade aos antimicrobianos, as estirpes de *Staphylococcus* isoladas a partir da bile e epitélio da vesícula biliar apresentou sensibilidade a: penicilina G, ceftriaxona, cloranfenicol e gentamicina. A observação de que a vesícula biliar comporta microrganismos em elevadas frequências atenta-nos para o fato de que o bovino possa ser um portador persistente de patógenos de grande importância em saúde pública.

Palavras-chave: Vesícula biliar de bovino. Microrganismos patogênicos. Portador persistente. Saúde pública.

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