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# Drug-resistant bacteria in frozen and fresh marine shrimp

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This study aimed to evaluate the bacteriological quality of frozen and fresh shrimp samples by *Salmonella* detection and the quantification of staphylococci and coliforms. Antimicrobial susceptibility profile of staphylococci isolates was also determined. A total of 30 shrimp samples commercialized in Sobral-CE - 15 fresh and 15 frozen, each one weighing 500 g - was analyzed. There was no contamination by *Salmonella* and/or coliform, but the *Staphylococcus* quantification showed that 12 samples (80.0%) of the frozen and 10 (66.7%) of the fresh shrimp presented a bacterial load above 3.0 $\log_{10}$ CFU $g^{-1}$ - a limit recommended by the current legislation in Brazil. 17 drug-resistant staphylococci strains were isolated, and the following antimicrobial resistance profiles were detected: monoresistance (n=4), cross-resistance to beta-lactam (n=4), and multidrug resistance to: Oxacillin+ampicillin+tetracycline (n=1), oxacillin+tetracycline+ penicillin+chloramphenicol+vancomycin (n=1). The findings indicate that frozen and fresh shrimps may act as vehicles for the spread of staphylococci resistant to drugs of clinical interest.

**Key words:** Enterobacteria, *Staphylococcus* drug-resistant, shrimp.

## INTRODUCTION

Seafood contaminated with foodborne bacterial pathogens is a worldwide problem. In this context, food outbreak cases involving the consumption of shrimp have been reported (Jiménez et al., 2011), serving as an alert to the need to ensure a more thorough quality of the product.

In shrimps, the most commonly used indicators for bacterial quality are the enteric bacteria (fecal coliforms and *Salmonella*) (Koonse et al., 2005) and *Staphylococcus* (Noor et al., 2014). Fecal coliforms and bacteria from the genus *Salmonella* are indicators of fecal contamination, and its occurrence in seafood is associated with food poisoning by infectious agents. On the other hand, the presence of staphylococci in food is associated with inadequate handling practices, causing food poisoning by intoxication.

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Other problem that has been associated with shrimp consumption is the emergence of drug-resistant microorganisms in these invertebrates. The data obtained from Nawaz et al. (2015) indicate that the use of antibiotics in shrimp aquaculture ponds may select bacteria resistant to these drugs. Furthermore, these authors alert to the fact that imported shrimp can be a reservoir of multiple antibiotic-resistant bacteria.

Food contamination with multidrug-resistant bacteria is considered a potential source for the wide dissemination of resistant-bacteria in communities (Le et al., 2015). Resistance to 16 drugs (ampicillin, ciprofloxacin, polymixin B, cefixime, amoxicillin, ceftriazone, penicillin, chloramphenicol, trimethoprime-sulfomethoxazole, gentamycin, nalidixic acid, kanamycin, vancomycine, erythromycin, tetracycline, streptomycin) in pathogenic bacteria isolated from shrimps was reported by Noor et al. (2014), indicating that these invertebrates can be vehicles in the transmission of drug resistant strains.

Thus, this study aimed to evaluate the bacteriological quality of frozen and fresh shrimp samples by (1) *Salmonella* detection, (2) quantification of staphylococci and coliforms, and (3) assessment of susceptibility profiles of staphylococci strains.

## MATERIALS AND METHODS

Samples of *Litopenaeus vannamei* shrimp (15 fresh and 15 frozen, each sample consisting of a shrimp pool weighing 500 g) were purchased at local retail markets in Sobral, Ceará, Brazil. All samples were stored in isothermal boxes and taken to the Laboratory for Bioprospecting and Applied Molecular Experimentation (NUBEM) at INTA College. The time gap between sampling and analysis took no longer than one hour.

### Preparation

For *Staphylococcus* analysis and coliform quantification, 25 g of each sample were aseptically weighed and mixed in 225 mL of saline solution at 0.85%. This homogenate corresponded to a $10^{-1}$ dilution, and it was the standard set for subsequent series of decimal dilutions up until $10^{-3}$ in a 0.85% saline solution, at a 1:9 ratio.

### Quantification and isolation of staphylococci

*Staphylococcus* Standard Plate Count (SPC) was performed in Agar Baird-Paker (BP - Difco) enriched with an egg yolk solution at 50 and 1% potassium tellurite, as described by Bennett and Lancette (2001). Colonies in the BP medium grown with typical *Staphylococcus* features (black, and with halo) were isolated in Brain Heart Infusion broth (BHI- Difco). All strains were subjected to biochemical screening and were identified as *Staphylococcus aureus* when presented the following profile; (1) Gram-positive cocci; (2) ability to coagulate rabbit plasma; (3) mannitol (+) in mannitol salt agar (10% NaCl); (4) oxidase (-) in oxidase strips (Laborclin); (5) acetoin production (+) in Voges-Proskauer. SPC calculation was performed by multiplying the colony count of *Staphylococcus* by its correspondent dilution, expressed as $\log_{10}$ colony forming units (CFU) per gram.

### Coliform quantification

Quantification of coliforms was performed by the multiple-tube fermentation method according to recommendations of Feng et al. (2002), using presumptive test and confirmed test for fecal coliform. For presumptive test, 1 mL aliquots from each dilution ($10^{-1}$ to $10^{-3}$) were inoculated into 3 lauryl tryptose broth tubes incubated at $35 \pm 0.5°C$. Confirmed test for fecal coliform was performed from inoculation of positive (fermentation of lactose) tubes to EC broth and incubated at $44.5 \pm 0.2°C$ for $24 \pm 2$ h.

### Salmonella detection

25 g of each sample were aseptically weighed and inoculated in lactose broth, with incubation for 24 h at 35°C. After incubation, selective enrichment was performed in Tetrathionate Broth (Difco) for 24 h at 35°C, followed by plating on MacConkey agar (Difco) and Brilliant Green Bile Agar. After this procedure, colonies with *Salmonella* characteristics were plated on tryptone soy agar medium (TSA) for biochemical screening in lysine iron agar, sulfide indole motility agar and triple sugar iron agar (Andrews et al., 2014).

### Antibiogram

*Staphylococcus* colonies isolated in TSA were selected and put to antimicrobial susceptibility testing using disk-diffusion technique in Mueller-Hinton agar. All colonies were suspended in a 0.85% saline solution until a 0.5 turbidity in the McFarland scale. This suspension was then homogenized, and colonies were plated with a sterile swab on Mueller-Hinton agar. After this procedure, the following antimicrobial discs were applied: Imipenem 10 μg (IMP); cefepime 30 μg (CPM); chloramphenicol 30 μg (CLO); streptomycin 10 μg (EST); ceftriaxone 30 μg (CTX); oxacillin 1 μg (OXA); tetracycline 30 μg (TET); gentamicin 10 μg (GEN); penicillin 10 μg (PEN); ciprofloxacin 30 μg (CRO); vancomycin 30 μg (VAN); ampicillin 10 μg (AMP). All plates were incubated at 35°C for 24 h. Diameter of the inhibition zone was then measured, and the strains were classified according to the standard established by CLSI (2012).

### Statistical analysis

Data were analysed by software GraphPad Prism 5.0. ANOVA followed by Student-Newman Keuls as *post hoc* test. The values of p<0.05 were considered statistically significant.

## RESULTS

*Salmonella* was not detected in any of the 30 samples analyzed: 15 samples of frozen shrimp and 15 of fresh shrimp. Index <3.0 MPN $mL^{-1}$ of fecal coliform was detected for all samples. The results for the quantification of coagulase-positive *Staphylococcus* (CPS) are shown in Figure 1. For frozen shrimp samples, an oscillation of 2.43 to 5.28 $\log_{10}$ CFU $g^{-1}$ was observed. In fresh shrimp, staphylococci population ranged from 2.19 to 5.39 $\log_{10}$ CFU $g^{-1}$. There was no significant statistical difference (p>0.05) between frozen shrimp versus fresh shrimp. Table 1 shows the results for antimicrobial resistance profiles of 76 staphylococci strains: 10 profiles were
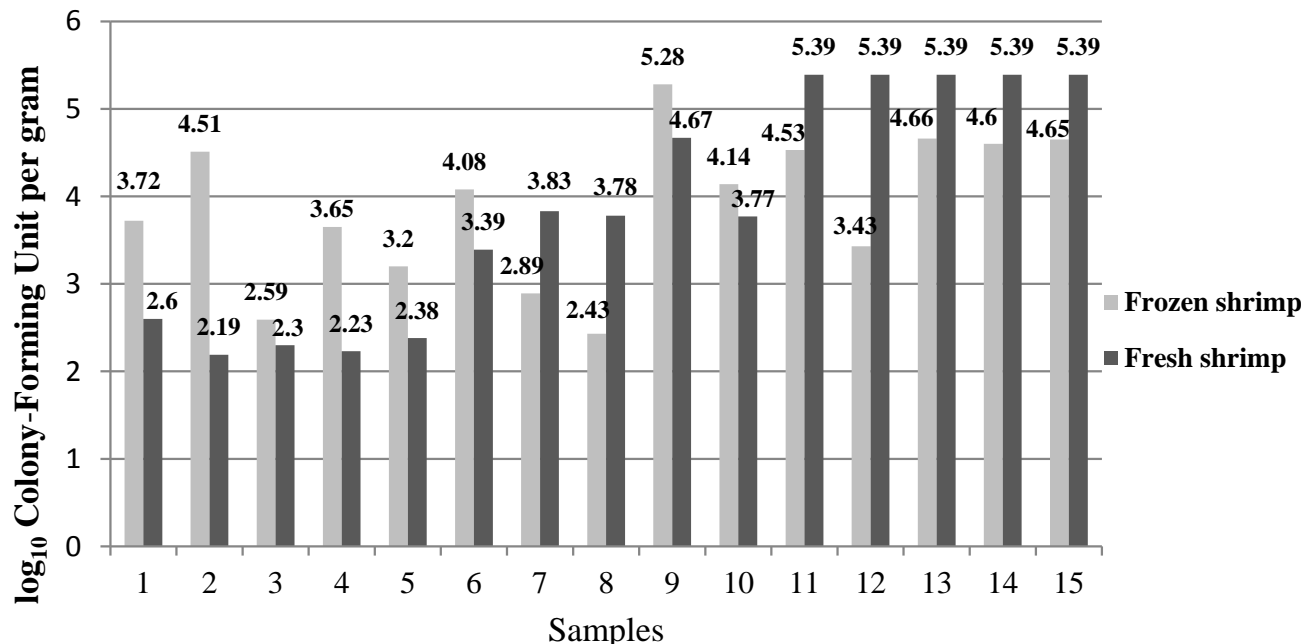
**Figure 1.** Quantification of *Staphylococcus* (log$_{10}$ Colony Forming Unit per gram) in frozen and fresh shrimp samples marketed in Sobral-CE.

detected, and we highlight the number of strains with monoresistance to oxacillin (n=5), cross-resistance to Oxa+Cpm (n=3), and multidrug resistance to Oxa+Amp+Tcy (n=1) and Oxa+Tcy+Pen+Clo+Van (n=1).

## DISCUSSION

The *Salmonella* data are in agreement with the Brazilian current legislation - Resolution RDC n° 12/2001 from the National Health Surveillance Agency (Brasil, 2001), which determines the absence of *Salmonella* in 25 g of frozen and fresh shrimps as a quality criteria. Absence of *Salmonella* and fecal coliforms indicates that samples of frozen and fresh shrimps (n=30) were not contaminated in the aquatic environment and/or during the handling process by feces of warm-blooded animals.

In the present study, the fact that no fecal indicator was not detected is in accordance with the findings of Koonse et al. (2005), who examined the prevalence of *Salmonella* and coliform bacteria in shrimp aquaculture farms, and found a significant relationship (p = 0.0342) between the log number of fecal bacteria and the probability of detecting *Salmonella* in any given sample.

On the other hand, *Salmonella* contamination in shrimp has been previously reported (Akiyama et al., 2011; Banerjee et al., 2012). In this context, Zhang et al. (2015) investigated the incidence of *Salmonella* in 730 samples aquaculture products from China, and found discordant results with this present study, since 217 (29.7%) of samples were positive for this bacteria. For these

authors, aquaculture products, including shrimps, can become sources of *Salmonella* by exposure to contaminated water or through processing practices. Absence of fecal coliform (FC) in both types of samples (fresh and frozen) also contrasts with recent studies. Parente et al. (2011) evaluated the bacteriological quality of 28 shrimp samples cultivated in Brazil, and in 22 samples a quantification of CF was detected, with oscillation of 3.6 to 2.1 × 10$^4$; only 6 samples had <3.0 MPN/CF. For the authors, the presence of FC in the shrimps is related to the quality of the water from the pond.

The results of *Staphylococcus* quantification (Figure 1) showed that 12 samples (80.0%) of frozen shrimp and ten (66.7%) sample units of fresh shrimp presented a bacterial load above 3.0 log$_{10}$ CFU g$^{-1}$; the recommended limit by Brazil (2001). These results serve as a warning about the quality of seafood, since the occurrence of coagulase-positive staphylococci in foods is associated with improper handling practices (Kadariya et al., 2014), indicating inadequate hygiene in any of the stages from capture to distribution of the end product to the consumer.

Comparing the values of the bacterial count of sample units, it is clear that the detection of staphylococcal rates above those permitted by the current legislation in Brazil was more frequent in frozen shrimp samples (80%). This may be related to the quality of raw material, since it is expected that the freezing decrease the microbial load on foods.

The detection of bacteria in frozen seafood is not an

**Table 1.** Antimicrobial resistance profile of staphylococci isolated from frozen and fresh shrimp.

| Resistance profile | Frozen shrimp (n=39) | Fresh shrimp (n=37) |
|---|---|---|
| Monoresistance | | |
| Oxa | 4 | 1 |
| Tcy | 1 | 1 |
| Cro | - | 1 |
| Pen | - | 1 |
| Cross-resistanceto beta-lactam | | |
| Oxa+Com | 2 | 1 |
| Oxa+Amp | - | 1 |
| Oxa+Amp+Pen | - | 1 |
| Oxa+Amp+Cpm+Ctx | - | 1 |
| Multidrugresistance | | |
| Oxa+Amp+Tet | - | 1 |
| Oxa+Tcy+Pen+Clo+Van | 1 | - |
| Total (%) | 8 (20.5) | 9 (24.3) |

n, Isolates number. Oxa, oxacillin1 µg; Tcy, tetracycline 30 µg; Cro, ciprofloxacin 30 µg; Pen, penicillin 10 UI; Cpm, cefepime 30 µg; Amp, ampicillin 10 µg; Ctx, ceftriaxone 30 µg; Clo, chloramphenicol30 µg; Van, Vancomycin 30 µg.

unusual fact. Noor et al. (2014) evaluated frozen shrimps in Bangladesh, and found samples contaminated with a huge bacterial load ($10^6$-$10^8$ CFU/g). Among the specific pathogens, staphylococci were detected. Noor Uddin et al. (2013) researched the bacterial flora in raw frozen cultured seafood (raw frozen cultured and wild-caught shrimp and fish) imported to Denmark and identified 6% of bacterial isolates as *Staphylococcus*.

In the present study, resistant staphylococci strains (n=17; 22.3%) were isolated from both types of shrimp (Table 1). This fact may be related to the indiscriminate use of antibiotics in shrimp farming. Intensified aquaculture includes the use of antimicrobials for disease control (Noor Uddin et al., 2013). For Duran and Marshall (2005), the occurrence of antibiotic-resistant bacteria in food products of animal origin is a potential health threat because resistance might be transferred among bacteria, and antibiotic-resistant pathogens may not respond to antibiotic treatments.

Detection of staphylococcal strains resistant to beta-lactams can be related to: β-lactamase acquisition, modification of penicillin-binding proteins, or acquisition of low-drug-affinity penicillin-binding proteins (Krupa et al., 2014).

Staphylococci resistant strains to penicillinase-stable penicillins- oxacillin (n=13) (Table 1) - may constitute a potential health risk for consumers, considering that oxacillin and methicillin resistant strains are resistant to all β-lactam agents (Matouskova and Janout, 2008). In this study, 5 strains resistant only to oxacillin were detected (Table 1). This may be explained by the fact that methicillin/oxacillin-resistant staphylococci are heterogeneous in their expression of resistance to β-lactam agents, and test conditions have a major effect on the expression (Brown, 2001).

Besides beta-lactams, tetracycline resistance was detected (Table 1). Tetracyclines (TCAs) are a broad spectrum of drugs that have been successfully used worldwide in both veterinary medicine and in aquaculture (Andersen et al., 2005). The data of this research related to tetracycline resistance are not an unusual fact. Tuševljak et al. (2013) researched the antimicrobial use and resistance in aquaculture through the opinion of aquaculture-allied professionals around the world. The questionnaire was administered to 604 professionals in 25 countries, and pointed out that the use of tetracycline was reported by 9% of respondents working with shrimp. Resistance to tetracycline in one or more species of bacteria was reported as 'frequent-to-almost always' for shrimp species by 36% of respondents.

Two strains presented as multi-drug resistant to Oxa+Amp+Tet (n=1) and Oxa+Tet+Pen+Clo+Van (n=1) (Table 1). Isolation of multidrug-resistant bacteria from aquaculture products has been reported (Zhang et al., 2012; Nawaz et al., 2015), emphasizing the importance of controlling the use of these drugs in the farming of aquatic organisms.

The findings of this study serve as a warning to the need of good handling practices implementation in all shrimp production stages, from capture to marketing, in order to ensure food safety for its consumers. In addition, the study of phenotypic profile of strains indicates that frozen and fresh shrimps may act as vehicles for the spread of staphylococci resistant to drugs of clinical significance. However, the determination of genetic origin (chromosomal or mobile genetic elements) by the new molecular methods is a vital aspect for research of bacterial drug resistance.

**Conflict of Interests**

The authors have not declared any conflict of interests.

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