

## Drug resistance, AmpC- $\beta$ -lactamase and extended-spectrum $\beta$ -lactamase-producing Enterobacteriaceae isolated from fish and shrimp

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### ABSTRACT

The present study aims to detect the production of extended-spectrum beta-lactamases (ESBL) by enterobacteria isolated from samples of fresh shrimp and fish obtained from the retail trade of the city of Sobral, Ceará State, Brazil. All bacterial isolates were submitted to identification and antimicrobial susceptibility testing using aminopenicillin, beta-lactamase inhibitors, carbapenem, 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> generation cephalosporins, and monobactam. Three types of beta-lactamases - ESBL, AmpC and KPC - were investigated. 103 strains were identified, and the most frequent species in shrimp and fish samples was *Enterobacter cloacae* (n = 54). All the strains were resistant to penicillin and more than 50% of the isolates were resistant to ampicillin and cephalothin. Resistance to three 3<sup>rd</sup> generation cephalosporins (cefotaxime, ceftriaxone and ceftazidime) and one fourth generation cephalosporin (cefepime) was detected in two isolates of *E. cloacae* from shrimp samples. Phenotypic detection of AmpC was confirmed in seven strains. The ESBL was detected in two strains of *E. cloacae* from shrimp samples. No strain showed KPC production. These data can be considered alarming, since food (shrimp and fish) may be carriers of enterobacteria resistant to drugs of clinical interest.

**KEYWORDS:** *Enterobacter cloacae*. Beta-lactam antibiotics. Food contamination. Antibiotic resistance. Food safety. Food-borne infections.

### INTRODUCTION

Enterobacteria are often associated with drug resistance and contamination of fish and shrimp<sup>1</sup>. Food contamination with multidrug-resistant bacteria, particularly extended-spectrum  $\beta$ -lactamase (ESBL)-producing Enterobacteriaceae, is considered a potential source for the wide dissemination of ESBL-producing bacteria in communities<sup>2</sup>.

Beta-lactam antibiotics are one of the main groups used to combat Gram-negative and Gram-positive bacteria and account for 60% of the antibiotics used worldwide for treatment of infectious diseases<sup>3</sup>. This group is characterized by the presence of the beta-lactam ring, which provides not only the mechanism of action, but also a low direct toxicity; since that acts on the cell wall and this is not present in human cells<sup>4</sup>. One of the major mechanisms of resistance presented by enterobacteria is the production of beta-lactamases. Several groups of these enzymes are widespread among almost all pathogenic species of Gram-negative bacteria. The main beta-lactamase producers are *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus*, *Providencia* and *Enterobacter*<sup>5,6</sup>.

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ESBL are encoded by genes located on chromosomes and/or mobile genetic elements such as plasmids, transposons and integrons, carrying resistance genes to other classes of antibiotics. The wide range of genes encoding the beta-lactamase are related to this mobility. The isolation of ESBL producing multi-resistant strains is a major cause of therapy failure in humans, leading to a considerable increase in morbidity of bacterial infections<sup>7</sup>.

The emergence of drug-resistant bacteria is a public health problem and threatens the effectiveness of drug treatments<sup>8</sup>. One of the factors contributing to the spread of this problem is the indiscriminate use of antimicrobials in animal production, which favors the selection of resistant bacteria with the potential to spread in the community through direct contact and consumption of contaminated food<sup>9</sup>.

Considering that drug-resistant Enterobacteriaceae are associated with food-borne infections via seafood consumption, this study aims to detect the production of ESBLs by enterobacteria isolated from fish and shrimp.

## MATERIAL AND METHODS

### Enterobacteriaceae isolation

Enterobacteria were isolated from samples of farmed shrimp *Litopenaeus vannamei* and farmed fish *Oreochromis niloticus* obtained in the retail trade in the city of Sobral, Ceará State, Brazil). Shrimp (n=5) and fish (n=5) samples, consisting of 500 g each, were placed in sterile bags, packed in boxes with ice and transported to the *Núcleo de Bioprospecção e Experimentação Molecular Aplicada* (NUBEM) of the Instituto Superior de Teologia Aplicada (INTA Faculty). The time between the sample collection and samples processing did not exceed one hour. All samples were weighed (50 g) and each sample was inoculated in 450 mL of Lactose Broth (Difco, USA) and incubated at 35 °C for 48 h. An inoculum of Lactose Broth growth was plated on MacConkey Agar (Difco, USA) and Brilliant Green Bile Agar (Difco, USA), incubated at 35 °C for 24 h. After the incubation period, 103 colonies were isolated in order to perform morphotypes and biochemical characterization.

### Morphotypes and biochemical characterization

All isolates were submitted to Gram staining and identified through their phenotypic profile using the Vitek 2 Gram-negative test card<sup>10</sup> (bioMérieux, France) in an automated instrument for identification (Vitek® 2).

### Antibiogram

The bacterial isolates (n = 103) were maintained in Tryptone Soy Agar (Difco) until the completion of the antibiogram test. The antimicrobial susceptibility profile was performed by disk diffusion technique using Mueller-Hinton Agar (MH-Difco, USA)<sup>11</sup>, and the following antimicrobials disks (Laborclin, Brazil) were tested: Ampicillin 10 µg, Amoxicillin/clavulanate 20/10 µg, Aztreonam 30 µg, Cefuroxime 30 µg, Cefepime 30 µg, Cefoxitin 30 µg, Cefotaxime 30 µg, Ceftriaxone 30 µg, Cefaclor 30 µg, Cefpodoxime 10 µg, Ceftazidime 30 µg, Imipenem 10 µg, Meropenem 10 µg. All strains were diluted in 0.85% saline to obtain turbidity equivalent to McFarland scale 0.5 and aliquots were seeded with swabs on MH agar plates, with subsequent application of antibiotic disks. Plates were incubated at 35 °C and interpretation of inhibition halos was made according to the CLSI<sup>11</sup>.

### AmpC phenotypic detection

AmpC production was confirmed by the disk approximation test and performed with isolates of the CESP group (*Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., *Providencia* spp.) susceptible to cefoxitin. To perform this technique, a cefoxitin disk (30 µg) (Laborclin, Brazil) was placed in a 20 mm plate center (center to center) away from a ceftriaxone disk (30 µg) (Laborclin, Brazil) and ceftazidime disk (30 µg) (Laborclin, Brazil). The plate was incubated at 35 °C for 18-24 h. Cefoxitin acted as an inducer of the AmpC enzyme and the positivity was considered when the flattening halo around the ceftriaxone and/or ceftazidime disk was observed<sup>12</sup>.

### ESBL phenotypic detection

The phenotypic screening for ESBL production was carried out through antibiogram for isolates identified as *E. coli*, *K. pneumoniae* and *K. oxytoca* with five substrates (Laborclin discs, Brazil): aztreonam 30 µg, cefotaxime 30 µg, cefpodoxime 10 µg, ceftazidime 30 µg, ceftriaxone 30 µg. Strains resistant to at least one of the antimicrobial agents used in the screening were used to confirmatory test for synergism approach disk or double-disk synergism<sup>12</sup>. In this test, amoxicillin/clavulanic acid disk (20 mg/10 mg) (Laborclin, Brazil) was placed in the center of the plate and 20 mm (center to center) away from an aztreonam disk (30 µg) and a ceftazidime disk (30 mcg). The plate was incubated at 35 °C for 18-24 h. The test was considered positive when an increase or distortion of any inhibition zone of marker and amoxicillin/clavulanic acid disk<sup>12</sup>.

## KPC phenotypic detection

For the KPC detection test, isolates with resistance to some third-generation cephalosporins (ceftazidime, ceftriaxone or cefotaxime) and carbapenem (imipenem or meropenem) were submitted to the modified Hodge test. To carry out this test, an inoculum of *E. coli* ATCC 25922 was seeded on the surface of a Mueller-Hinton Agar (Difco, USA) plate and a 10-μg meropenem disk (Laborclin, Brazil) was placed in the center of the plate. Thus, three to five newly cultured (24 h) colonies from the sample were seeded from the center of the meropenem disk to the periphery of the Petri dish in order to draw an imaginary line of 20 to 25 mm. After incubation at 35 °C for 16-20 h the test was considered positive when there was growth of *E. coli* ATCC® at 25922 in the inhibition zone of meropenem<sup>12</sup>.

## RESULTS

Table 1 shows the diversity of 103 enterobacteria strains among the isolates from shrimp (*L. vannamei*) and fish (*O. niloticus*). *E. cloacae* (n = 54) was the most frequently isolated species from shrimp and fish samples, followed by *K. pneumoniae* (n = 22) and *E. coli* (n = 14).

It was observed that all strains were resistant to penicillin and more than 50% of the isolates were resistant to ampicillin and cephalothin. Resistance to third (cefotaxime, ceftriaxone and ceftazidime) and fourth-generation (cefepime) cephalosporins was detected in two isolates of *E. cloacae* from shrimp (Table 2).

AmpC phenotypic detection was only confirmed in *E. cloacae* (n = 3) and *C. freundii* (n = 2) from shrimp and in *E. cloacae* (n = 2) and *C. braakii* (Table 3). ESBL was

**Table 1** - Enterobacteria species isolated from shrimp *Litopenaeus vannamei* and fish *Oreochromis niloticus*

Specie	Shrimp		Fish		Total of strains
	n	Sample of strains origin	n	Sample of strains origin	
<i>Enterobacter cloacae</i>	25	1, 2, 3, 4, 5	29	1, 2, 3, 4, 5	54
<i>Klebsiella pneumoniae</i>	14	1, 2, 3, 5	8	1, 2, 3, 5	22
<i>Escherichia coli</i>	10	2, 3, 4, 5	4	1, 2, 3	14
<i>Citrobacter freundii</i>	3	1	2	1, 2	5
<i>Enterobacter aerogenes</i>	2	5	-	-	2
<i>Raoutella planticola</i>	-	-	2	2	2
<i>Escherichia hermannii</i>	-	-	1	1	1
<i>Citrobacter braakii</i>	-	-	1	2	1
<i>Providencia stuartii</i>	-	-	1	1	1
<i>Serratia marcescens</i>	-	-	1	3	1
Total	54	-	49	-	103

\*n= number of strains identified.

**Table 2** - Distribution of the enterobacteria strains (n=103) according to beta-lactam resistance

Specie	Drugs											
	Pen	Amp	Cpl	Cfo	Cfc	Crx	Ctx	Caz	Cro	Cpm	Atm	lpm
<i>E. cloacae</i>	54	28	42	40	7	-	2	2	1	2	3	1
<i>K. pneumoniae</i>	22	13	5	6	1	-	-	-	-	-	-	-
<i>E. coli</i>	14	6	1	-	-	-	-	-	-	-	-	-
<i>C. freundii</i>	5	1	3	2	-	-	-	-	-	-	-	-
<i>E. aerogenes</i>	2	2	-	-	-	-	-	-	-	-	-	-
<i>R. planticola</i>	2	2	1	1	-	-	-	-	-	-	-	-
<i>E. hermannii</i>	1	-	-	-	-	-	-	-	-	-	-	-
<i>C. braakii</i>	1	-	1	1	-	-	-	-	-	-	-	-
<i>P. stuartii</i>	1	-	1	-	-	-	-	-	-	-	-	-
<i>S. marcescens</i>	1	-	-	-	-	1	-	-	-	-	-	-
Total (n)	103	52	54	50	8	1	2	2	1	2	3	1
%	100	50.5	52.4	48.5	7.7	0.97	1.9	1.9	0.97	1.9	2.9	0.9

\* -: no resistant strain. Penicillin (Pen), Ampicillin (Amp), Cephalothin (Cpl), Cefoxitin (Cfo), Cefaclor (Cfc), Cefuroxime (Crx), Cefotaxime (Ctx), Ceftazidime (Caz), Ceftriaxone (Cro), Cefepime (Cpm), Aztreonam (Atm) and Imipenem (lpm).

**Table 3** - Number of enterobacteria strains according to the expression of AmpC beta-lactamase

Specie	Shrimp		Fish	
	evaluated strains	positive strains	evaluated strains	positive strains
<i>Enterobacter cloacae</i>	11	3	3	2
<i>Enterobacter aerogenes</i>	2	-	-	-
<i>Citrobacter freundii</i>	3	2	-	-
<i>Citrobacter braakii</i>	-	-	1	1
<i>Serratia marcescens</i>	-	-	1	-
<i>Providencia stuartii</i>	-	-	1	-
Total (%)	16 (100)	5 (31.2)	6 (100)	3 (50)

detected in two strains of *E. cloacae* from shrimp. No strain showed KPC production.

## DISCUSSION

In the present study, 49 (89%) of the shrimp isolates and 41 (83%) of the fish isolates corresponded to *E. cloacae*, *K. pneumoniae* and *E. coli* species (Table 1). The isolation of enterobacteria from shrimp and fish has already been reported<sup>13,14</sup>, indicating fecal contamination, since these microorganisms are not part of the normal microbiota of these animals.

*E. cloacae* has already been isolated from aquatic organisms<sup>15,16</sup> and stands out as a human pathogen present in nosocomial outbreaks<sup>17</sup>. In addition, *E. cloacae* with the ability to produce biofilm was isolated from fish and seafood, a fact that favors resistance to antibiotics<sup>16</sup>.

The presence of *K. pneumoniae* in fish and shrimp samples (Table 1) serves as an alert and the health risks presented by these strains should not be underestimated. Some studies corroborate the present investigation since they reported the isolation of these species from fish, seafood and other types of food<sup>18,19</sup>.

The third most frequently isolated microorganism in the present study was *E. coli* (Table 1) and it is known that this species has been associated with food-borne infections and was isolated from fish and aquatic environments. It is important to note that fish contaminated by this bacterium is a risk factor for consumers<sup>20-22</sup>.

In addition, some studies have reported the presence of *C. freundii*<sup>23</sup> and *C. braakii*<sup>24</sup> in fish, corroborating data of the present study. This genus, generally regarded as a commensal bacterium of the human intestinal microbiota, can cause opportunistic infections. It has been associated with nosocomial, urinary and respiratory infections<sup>25</sup>.

In the present study, a strain of *R. planticola* (Table 1) was also isolated from a fish sample. This species has already been isolated from herbs, vegetables, water and

soil, but rarely from animals. This species is responsible for infections such as pneumonia, bacteremia in cancer patients or in those with a depressed immune system. *R. planticola* converts histidine to histamine and may produce symptoms of poisoning when undercooked seafood is consumed<sup>26,27</sup>.

*P. stuartii*, another bacterium isolated from fish sample (Table 1), usually causes opportunistic infections and is associated with nosocomial outbreaks<sup>28</sup>. Khunthongpan et al.<sup>29</sup> isolated this strain from processed wastewater from seafood.

Also in this sense, occurrence of a *S. marcescens* (Table 1) strain was observed in a fish sample. This bacterium is an important opportunistic pathogen associated with serious infections. Recently, in Brazil, Cayô et al.<sup>30</sup> reported an outbreak of septic shock associated with this strain. *S. marcescens* has been isolated from frozen shrimp and fish and may be associated with fish deterioration<sup>31</sup>.

Incidence of beta-lactam-resistant Enterobacteriaceae in aquatic organisms (shrimp and fish) observed in the present study (Table 2) may be related to the indiscriminate use of antibacterial drugs in the culture. In the present study, more than 50% of the strains were resistant to ampicillin. Some enterobacteria are naturally resistant to aminopenicillins or may acquire beta-lactam resistance genes, which results in ampicillin resistance<sup>26,27</sup>.

Fifty-four (52.4%) strains presented resistance to cephalothin (Table 2). These data are in agreement with findings in the literature, since strains isolated from fish have been showing resistance to this first-generation cephalosporin<sup>32,33</sup>. In addition, cefoxitin- and cefuroxime-resistant bacteria have also been isolated from food<sup>34,35</sup>.

In the present study, resistant strains of third and fourth-generation cephalosporins were detected (Table 2). These data are alarming, since these drugs are used in clinical practice. Liu et al.<sup>36</sup> detected enterobacteria with resistance to third-generation cephalosporins, suggesting that resistance was associated with class 1 integrons. Resistance to cefepime has been reported in strains of hospital origin<sup>37</sup>.

The present study also demonstrated that 3 (2.9%) of the isolates were resistant to aztreonam (Table 3). Bacteria resistant to this drug have been isolated from shrimp farms<sup>38</sup>. On the other hand, resistance to imipenem, that has also been observed in the present research (Table 2), has been reported in the literature in clinical isolates<sup>39</sup>.

AmpC beta-lactamase is associated with the bacterial ability to resist cephalosporins and beta-lactamase inhibitors. In the present research, this enzyme was detected in the species *E. cloacae*, *C. freundii* and *C. braakii* (Table 3). A survey carried out in a Vietnamese fish market revealed a high contamination rate of shrimp (49.1%) and fish (36.6%) by enterobacteria. The authors showed that all strains of the Enterobacteriaceae family showed to be AmpC enzyme carriers and showed resistance to at least 6 classes of antimicrobials, including beta-lactams. The presence of these multiresistant multi-resistant strains is a concern for public health and suggests that the use of antimicrobial agents in fish culture is to be strictly controlled<sup>1</sup>.

In the present study, ESBL was detected in two strains of *E. cloacae* from shrimp, only. Le *et al.*<sup>2</sup> detected *E. coli* producing ESBL in shrimp. The authors warn of the severity of these results, since this fact requires monitoring and effort by the monitoring agencies to control the spread of ESBL-producing bacteria in the community.

Janecko *et al.*<sup>40</sup> detected bla<sub>NDM-1</sub> and bla<sub>IMI-type</sub> resistance genes encoding beta-lactamases in *E. cloacae* and *E. aerogenes* isolated from fish and shrimp. This research suggests that, in addition to the probable fish contamination by water, the presence of beta-lactamases may be associated with fish exposure during transport.

In the present study, no strain expressed KPC. This fact may be related to the limitation of the phenotypic test used (Hodge test), which, although already used for KPC detection<sup>12</sup>, is not recommended by CLSI to detect this enzyme. In addition, the lack of KPC expression may also be related to the high number of strains sensitive to monobactam and carbapenems. KPC is an extended-spectrum beta-lactamase capable of hydrolyzing carbapenems and other beta-lactam antibiotics. Their detection is crucial because they are often associated with total or partial antibiotic resistance<sup>41</sup>. This enzyme has already been detected in seafood isolates that showed cross-resistance to penicillins, cephalosporins, monobactam and carbapenem<sup>41,42</sup>.

The results indicate that aquatic organisms (fish and shrimp) may constitute sources of enteric bacteria resistant to beta-lactams. These data reveal the need to establish legislation regulating the use of antimicrobials in aquaculture activities, and it is suggested that sanitary authorities urgently sanction control measures for use

of antimicrobials in fish and shrimp farming. It is also emphasized that this study presents as a limitation the absence of information about the genetic characterization of strains by PCR, therefore, there is no data of distribution of the genes-bla<sub>CTX-M</sub>, bla<sub>TEM</sub> and bla<sub>SHV</sub> among the 103 isolates.

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