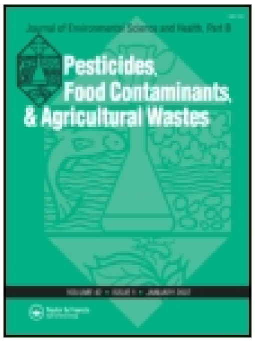


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# Antimicrobial susceptibility of *Escherichia coli* isolated from shrimp (*Litopenaeus vannamei*) and pond environment in northeastern Brazil

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# Antimicrobial susceptibility of *Escherichia coli* isolated from shrimp (*Litopenaeus vannamei*) and pond environment in northeastern Brazil

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This study aimed to test the susceptibility of *Escherichia coli* strains isolated from the water, bottom sediments and individuals cultivated in shrimp farm ponds, to antibiotics belonging to different families, namely B-Lactams: Imipenem (IPM; 10 $\mu$g), Ampicillin (AMP; 10 $\mu$g), Cephalothin (CEP; 30 $\mu$g), Cefoxitin (FOX; 30 $\mu$g), Ceftriaxone (CRO; 30 $\mu$g); Tetracycline: Tetracycline (TCY; 30 $\mu$g); Aminoglycosides: Gentamicin (GEN; 10 $\mu$g), Amikacin (AMK; 30 $\mu$g); Chloramphenicol: Chloramphenicol (CHO; 30 $\mu$g); Fluoroquinolones: Ciprofloxacin (CIP; 5 $\mu$g); Nitrofurans: Nitrofurantoin (NIT; 300 $\mu$g); Sulfonamides: Trimethoprim-Sulfamethoxazole (SXT; 30 $\mu$g); Quilononas: Nalidixic Acid (NAL; 30 $\mu$g). In the laboratory, the method of dissemination (Test Kirby-Bauer) was performed in order to fulfill the antibiogram tests. The results showed high indices of resistance to Imipenem, Cephalothin and Ampicillin. Chloramphenicol, Nitrofurantoin, Cefoxitin, Ceftiaxone and Ciprofloxacin have displayed the highest index of sensitive strains. The antibiotic resistance index (ARI) and the multiple resistance index (MAR) varied within the ranges of 0.068–0.077 and 0.15–0.39, respectively. More than 90.5% of strains of *Escherichia coli* showed a variety of resistance profiles to the tested antibiotics. The high indices of resistance may be a consequence of indiscriminate use of antibiotics, but also the transfer of resistance through mobile genetic elements found in shrimp farms.

**Keywords:** Susceptibility; *Escherichia coli*; antibiotics; *Litopenaeus vannamei*; shrimp farm.

## Introduction

Shrimp farming plays an important role in the Brazilian seafood industry, both in the perspective of the organization of the productive chain and as a source of jobs, income and foreign currency, and has made a positive contribution to the establishment of a new and permanent productive structure in the rural coastal zone.[1] However, shrimp farming is becoming increasingly vulnerable to infectious diseases caused by protozoa, fungi, bacteria and virus due to the ease with which pathogens spread in culture environments.[2]

In addition to stress, which has been shown to trigger disease in shrimp,[3] the question of farm water quality is of much concern to microbiologists. Water quality may be determined by screening for thermotolerant coliforms, the most important of which (70%) is *Escherichia coli*.[4] The presence of *E. coli* in water or food is an indicator of fecal contamination and exposure of consumers to potentially pathogenic microorganisms.[5]

While most strains of *E. coli* are harmless to man, some strains are virulent and may be life-threatening.[5–6]

Microbiological water quality has become a public health concern mainly due to the emergence of pathogenic bacteria resistant to antibiotics. The generalized use of antibiotics in animal husbandry is known to promote the development of resistant bacteria or resistance genes which may be transferred to bacteria capable of infecting humans.[7]

Thus, the number of resistant bacterial strains found in aquatic environments has been growing over the past decades as a result of the indiscriminate use of antibiotics in animal or human prophylaxis and treatment, as well as in food production. The process is also associated with the dissemination of plasmids containing antibiotic-resistance genes that render microorganisms genetically more flexible with regard to adjustment and survival in hostile environments.[8]

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The objective of the present study was to test the antimicrobial susceptibility of *E.coli* isolated from shrimp culture environments and shrimps (*Litopenaeus vannamei*) in Northeastern Brazil.

## Materials and methods

### *Origin and preparation of strains*

The study included 21 *E. coli* strains isolated from shrimp and shrimp pond water and bottom sediments from three farms located in the estuaries of the Jaguaribe river, the Acaraú river and the Coreaú river, respectively (coastal zone of the state of Ceará). Sampling was performed during the dry season (June, July, August and September) and the rainy season (February and March). The *E. coli* strains were isolated from pond water (n=10), pond sediments (n=6) and shrimp tissue (n=5). Currently the strains are stored in agar tubes in a B.O.D. incubator at 23°C at *Laboratório de Microbiologia Ambiental e do Pescado* (LABOMAR/UFC) in Fortaleza, Ceará, Brazil. Prior to antibiogram testing, the strains were submitted to culture purity assessment in Eosin Methylene Blue (EMB) agar medium and reidentification with IMViC, as described by Mehlman, Andrews and Wentz.[9]

### *Antibiogram*

Mueller-Hinton agar was used as growth medium for antibiotic susceptibility testing, as recommended by the Clinical and Laboratory Standards Institute (CLSI).[10] The antibiotic discs were cold-stored at 2–8°C and used before their expiry date. Prior to testing, the discs were left at room temperature for two hours.

The study used antibiotics sensitivity disc (LABCLIN, Paraná, Brazil) belonging to the following families: a) the $\beta$-lactam family: imipenem (IPM; 10$\mu$g), ampicillin (AMP; 10$\mu$g), cephalothin (CEP; 30$\mu$g), cefoxitin (FOX; 30$\mu$g) and ceftriaxone (CRO; 30$\mu$g); b) the tetracycline family: tetracycline (TCY; 30$\mu$g); c) the aminoglycoside family: gentamicin (GEN; 10$\mu$g) and amikacin (AMK; 30$\mu$g); d) the chloramphenicol family: chloramphenicol (CHO; 30$\mu$g); e) the fluoroquinolone family: ciprofloxacin (CIP; 5$\mu$g); f) the nitrofuran family: nitrofurantoin (NIT; 300$\mu$g); g) the sulfonamide family: trimethoprim-sulfamethoxazole (SXT; 30$\mu$g); and h) the quinolone family: nalidixic acid (NAL; 30$\mu$g), following recommendations of the CLSI.[10] The initial inoculum was standardized by spectrophotometry (Micronal, model B542) at 625nm and spread on Mueller-Hinton agar forming a layer on which the antibiotic discs were placed. After incubation at 35°C for 24 hours, the bacterial growth inhibition zone, if any, was measured using a caliper-square.

### *The antibiotic resistance index (ARI) and the multiple antibiotic resistance (MAR) index*

Based on the findings from the antibiogram, the antibiotic resistance index (ARI) and,[11] for strains resistant to more than one drug, the multiple antibiotic resistance index (MAR) were calculated. A MAR value above 0.2 indicates multiresistance.[12]

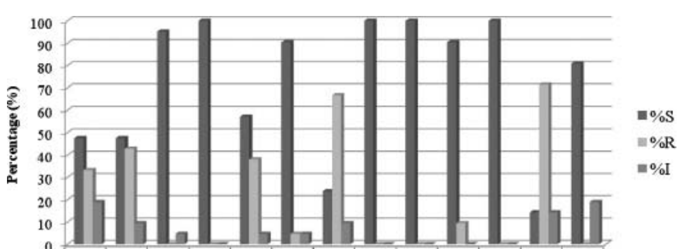
### *Plasmid curing*

Strains resistant to antibiotics from more than one family were submitted to plasmid curing with 100 $\mu$g/mL acridine orange in Luria-Bertani broth supplemented with 0.85% NaCl, as described by Molina-Aja et al.[13] After culture in this medium under continuous shaking at 30°C for 24 hours, the strains were again submitted to antibiogram testing with the drugs they were originally resistant to, in order to establish whether the resistance was related to plasmid or chromosomal DNA.

## Results and discussion

Figure 1 shows the percentages of sensitivity/resistance to antibiotics of 21 *E. coli* strains isolated shrimp pond water and sediments and samples of shrimp tissue (*Litopenaeus vannamei*).

A significant percentage of the strains (42.86%) were resistant to AMP. The drug belongs to the ß-lactam family and acts by inhibiting cell wall synthesis–a commonly employed principle in medical care.[14]




| | TCY | AMP | NAL | CHL | SXT | NIT | CEP | FOX | CRO | GEN | CIP | IMP | AMK |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| %S | 47.62 | 47.62 | 95.24 | 100 | 57.14 | 90.48 | 23.81 | 100 | 100 | 90.48 | 100 | 14.29 | 80.95 |
| %R | 33.33 | 42.86 | 0 | 0 | 38.1 | 4.76 | 66.67 | 0 | 0 | 9.52 | 0 | 71.43 | 0 |
| %I | 19.05 | 9.52 | 4.76 | 0 | 4.76 | 4.76 | 9.52 | 0 | 0 | 0 | 0,00 | 14.29 | 19.05 |

Antimicrobial Agentes

**Fig. 1.** Percentages of sensitivity/resistance to antibiotics of *E. coli* strains isolated from shrimp pond water and sediments and samples of shrimp tissue (*Litopenaeus vannamei*) from three farms in Northeastern Brazil. TCY=tetracycline, AMP=ampicillin, NAL=nalidixic acid, CHL=chloramphenicol, SXT= trimethoprim-sul-famethoxazol e, NIT=nitrofurantoin, CEP=cephalothin, FOX=cefoxitin, CR O =ceftriaxone, GEN=gentamicin, CIP=ciprofloxacin, IMP=imipenem, AMK =amikacin. S=Sensitive; I=Intermediate; R=Resistant.

Approximately 70% (15) of the *E. coli* strains isolated from the farming environment were resistant to IPM, another member of the $\beta$-lactam family. However, IPM has a greater antimicrobial spectrum and potential than AMP, and is the only carbapenem available for clinical use in Brazil, the US and Europe.[15] In a study evaluating fecal contamination in the oyster species *Crassostrea rhizophorae* and in pond water from an oyster farm located in the estuary of the Pacoti river (Eusébio, Ceará), Vieira et al.[16] found 80% of the *E. coli* strains isolated from the water and 100% of the strains isolated from the muscle and internal liquid to be resistant to IMP. This supports our findings of 71.43% resistance to IMP, suggesting the respective plasmids are widely disseminated in the environment. According to Hang et al.[17] some bacteria actually possess ß-lactamase-encoding chromosomal genes capable of hydrolyzing IMP, thereby making them resistant to treatment with this drug.

The high rate of resistance among strains from the environment is suggestive of widespread use of inadequate doses of antibiotics resulting in selective pressures on bacterial populations in the microbiota. In aquatic environments the situation is aggravated by the possibility of horizontal transference of genes between bacteria of the same or even different species and genera. In addition, in intensive farming systems with high stocking rates, antibiotics are commonly used to promote growth or for prophylaxis. As a result, aquatic bacteria around the world are becoming increasingly resistant to antimicrobial drugs, exposing both stocks and human consumers to serious health risks.[18]

In a study testing the antimicrobial susceptibility of bacteria isolated from various tilapia (*Oreochromis niloticus*) culture systems, Carneiro et al.[19] found species of the family Enterobacteriaceae to be resistant to AMP, TCY, NIT and SUT, and sensitive to CHO and GEN. According to Hölmstrom et al.[20] prophylaxis with antibiotics is now a common practice in aquaculture, especially in developing countries where the use of drugs in aquatic environments has not yet been regulated. However, many farmers are unaware that indiscriminate use of antibiotics can lead to changes in the culture microbiota. The presence of residual antibiotics favors the selection of resistant bacteria which may enter the human food chain by way of contaminated seafoods.[21]

In the present study all *E. coli* strains were susceptible to FOX, CRO, CIP, CHO, NAL, GEN and NIT, suggesting these drugs are not the treatment of choice on local shrimp farms.

Table 1 shows the resistance profile and respective MAR values of each *E. coli* strain tested. Seven (33.33%) of the isolated strains were resistant to five antibiotics. Five multiple-resistance profiles were observed among the strains isolated from the shrimp farm on the Acaraú river: (CEP, IMP), (NIT, CEP, IMP), (AMP, CEP, GEN, IMP), (TCY, AMP, SXT, CEP, IMP) and (AMP, SXT, CEP). Samples from the farms on the Coreaú river and the Jaguaribe river yielded one profile each: (CEP, IMP) and (TCY, AMP, SXT, CEP, IMP), respectively.

Eight of the strains isolated from pond water samples were resistant to at least one of the antibiotics tested while seven displayed intermediate-level resistance.

Aizemberg et al.[22]analyzed the antibiotic resistance profile of *E. coli* strains from the Monjolinho river and tributaries near São Carlos (São Paulo) and reported high percentages of resistance to CEP (53.2%), TCY (31.2%) and AMP (22%), associated with serious potential risks to human health through the contamination of water bodies by discharge from farms and human activities. In fact, resistant bacteria have been detected in a variety of environments, including domestic sewers, drinking water, rivers and lakes.[23]

In a study testing 98 *E. coli* strains from stormsewer and seawater samples from the coast of Natal (Rio Grande do Norte), Cardonha et al.[24]found 28 strains with multiple resistance to NAL, TCY and SXT. According to the authors, studies on resistant *E. coli* strains in the aquatic environment must take into account that these microorganisms are the product of previous selection. Strains with intermediate-level resistance (47.6% of the strains tested in the present study) are of particular concern. They behave like sensitive organisms but may have a negative impact on treatment efficacy. In other words, some of the bacteria in the microbiota may be particularly sensitive to a given antibiotic while others are genetically immune.[25]

Six of the strains isolated from pond sediments displayed antibiotic resistance, especially to AMP and SXT (42.86% and 38.1%, respectively) (Fig. 1). Likewise, Chelossi et al.[26] reported all Gram-negative strains isolated from their samples of fish pond sediments to be resistant to AMP and SXT.

According to Tendencia and de la Peña,[27] little is presently known about the occurrence of resistant bacteria in sediments of the areas surrounding marine aquaculture systems and their relation to the composition of local bacterial communities.

Five of the *E. coli* strains isolated from shrimp tissue were found to be resistant. Of these, four came from the farm on the Acaraú river and displayed a multiresistance profile identical to the profile of a strain (EC10) isolated from pond water from the same farm. The fifth strain, which was isolated from shrimp reared on the farm on the Coreaú river, was resistant to CEP and IMP (Table 1). The use of antibiotics for prophylaxis in shrimp culture affects the bacterial density in the ponds and the frequency of resistance to antimicrobial drugs. The antibiotics mixed in the shrimp feed may not be completely absorbed by the digestive tract and so become excreted in the pond water where they contribute to the selection of resistant bacteria in both microbiota and livestock.[28]

**Table 1.** Resistance profiles and multiple antibiotic resistance indices (MAR) of 21 *E. coli* strains isolated from shrimp pond water and sediments and samples of shrimp tissue (*Litopenaeus vannamei*) from three farms in Northeastern Brazil.

| *Sample* | *Sampling location* | *Strain* | *Profile* | | | *MAR* |
|---|---|---|---|---|---|---|
| | | | *R* | *I* | *S* | |
| Pond water | Acaraú | EC1 | IMP | TCY, AMP, AMK | NAL, CHL, SXT, NIT, CEP, FOX, CRO, GEN, CIP | – |
| | | EC2 | * | * | TCY, AMP, NAL, CHL, SXT, NIT, CEP, FOX, CRO, GEN, CIP, IMP, AMK | – |
| | | EC3 | CEP, IMP | TCY, AMP | NAL, CHL, SXT, NIT, FOX, CRO, GEN, CIP, AMK | 0.15 |
| | | EC4 | IMP | TCY, CEP | AMP, NAL, CHL, SXT, NIT, FOX, CRO, GEN, CIP, AMK | – |
| | | EC5 | AMP, CEP, GEN, IMP | AMK | TCY, NAL, CHL, SXT, NIT, FOX, CRO, CIP | 0.31 |
| | | EC6 | NIT, CEP, IMP | SXT, AMK | TCY, AMP, NAL, CHL, FOX, CRO, GEN, CIP | 0.23 |
| | | EC7 | IMP | CEP | TCY, AMP, NAL, CHL, SXT, NIT, FOX, CRO, GEN, CIP, AMK | – |
| | | EC8 | * | * | TCY, AMP, NAL, CHL, SXT, NIT, CEP, FOX, CRO, GEN, CIP, IMP, AMK | – |
| | | EC9 | GEN | IMP | TCY, AMP, NAL, CHL, SXT, NIT, CEP, FOX, CRO, CIP, AMK | – |
| | | EC10 | TCY, AMP, SXT, CEP, IMP | * | NAL, CHL, NIT, FOX, CRO, GEN, CIP, AMK | 0.39 |
| Pond sediment | | EC11 | CEP | TCY, NAL, IMP | AMP, CHL, SXT, NIT, FOX, CRO, GEN, CIP, AMK | – |
| | | EC12 | AMP, SXT, CEP | * | TCY, NAL, CHL, NIT, FOX, CRO, GEN, CIP, IMP, AMK | 0.23 |
| | | EC13 | CEP, IMP | * | TCY, AMP, NAL, CHL, SXT, NIT, FOX, CRO, GEN, CIP, AMK | 0,15 |
| | Jaguaribe | EC14 | TCY, AMP, SXT, CEP, IMP | - | NAL, CHL, NIT, FOX, CRO, GEN, CIP, AMK | 0.39 |
| | | EC15 | TCY, AMP, SXT, CEP, IMP | NIT | NAL, CHL, FOX, CRO, GEN, CIP, AMK | 0.39 |
| | Coreaú | EC16 | IMP | * | TCY, AMP, NAL, CHL, SXT, NIT, CEP, FOX, CRO, GEN, CIP, AMK | – |
| Shrimp tissue | Acaraú | EC17 | TCY, AMP, SXT, CEP, IMP | * | NAL, CHL, NIT, FOX, CRO, GEN, CIP, AMK | 0.39 |
| | | EC18 | TCY, AMP, SXT, CEP, IMP | * | NAL, CHL, NIT, FOX, CRO, GEN, CIP, AMK | 0.39 |
| | | EC19 | TCY, AMP, SXT, CEP, IMP | AMK | NAL, CHL, NIT, FOX, CRO, GEN, CIP | 0.39 |
| | | EC20 | TCY, AMP, SXT, CEP, IMP | * | NAL, CHL, NIT, FOX, CRO, GEN, CIP, AMK | 0.39 |
| | Coreaú | EC21 | CEP, IMP | * | TCY, AMP, NAL, CHL, SXT, NIT, FOX, CRO, GEN, CIP, AMK | 0.15 |

Table 1 shows the ARI and MAR values for each sampling location. Strains isolated from different shrimp farms differed with regard to resistance profile. Thus, strains from the farms on the Jaguaribe river and Coreaú river displayed higher ARI values (0.077) than strains from the farm on the Acaraú river (0.068). The observed indices suggest the risk of dissemination of resistant genes within the bacterial communities surveyed.

Indices of multiple antibiotic resistance to at least two drugs ranged from 0.15 to 0.39. MAR values were similar for the three farms but differences were observed in resistance profile. The claim of Kaspar et al.[29] that samples with similar MAR values also have similar resistance profile was not borne out in our study. Of the 21 strains collected for this study, 47.46% displayed MAR values of ≥0.2, indicating multiresistance. Such a high percentage of multiresistant bacteria suggests dissemination of mobile genetic elements or selection of mutant strains.

The presence of significant numbers of multiresistant bacteria in the aquatic environment represents a considerable ecological and public health concern and calls for further investigations into the factors determining resistance in different bacterial species and the possibility of transmission of resistant genes to pathogens capable of infecting human consumers of seafoods.[30]

In our study the phenotype of most strains was found to be of chromosomal origin. Consequently, antibiotic resistance was generally not affected by plasmid curing. The exceptions were a strain isolated from pond water from the farm at Acaraú (EC3) and a strain isolated from shrimp reared on the farm at Coreaú (EC21), which lost their resistance to CEP after plasmid curing. According to Madigan et al.[18] in most cases antibiotic resistance mediated by chromosomal genes arises because of a modification of the target of antibiotic action, in contrast with R-plasmid-mediated resistance which is due to genes encoding new enzymes that inactivate the drug or prevent uptake of the drug or actively pump it out.

Environmentalists, government agencies and researchers have expressed concern with the results of the indiscriminate use of antibiotics in aquaculture. The use of antibiotics should be better controlled to minimize the risk of transference of resistant genes between pathogenic bacteria in the microbiota of both livestock and the environment.

## Conclusion

*E. coli* strains sampled from three shrimp farms in Northeastern Brazil displayed high rates (90.5%) of antibiotic resistance. Strains isolated from pond water and sediments were resistant to IMP, CEP, AMP and TCY. The study shows that resistant genes are presently circulating on shrimp farms in the region.

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