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Prevalence and Antibiotic Susceptibility of Gram-negative Aerobic Bacteria Cultured from the Intestine and Hepatopancreas of Blue Land Crab (*Cardisoma guanhumi*) in Grenada, West Indies

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Authors' contributions

This work was carried out in collaboration between all authors. Authors VAA, RP, RS, and HH designed the study. Authors RS and RP managed the collection of all the samples. Authors VAA, VMB, and RP managed the analyses and literature searches. Authors VAA and HH wrote the protocol and wrote the first and final drafts of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate the prevalence of aerobic bacteria associated with the intestine and hepatopancreas of blue land crab and the susceptibility of the bacteria to a panel of antimicrobials that included some drugs used for the treatment of bacterial infections in the human and veterinary clinics in Grenada.
Study Design: The tested crabs were collected during a three month period from November 2011 to February 2012 from six parishes of Grenada and analyzed in the bacteriology lab in the Pathobiology Department, School of Veterinary medicine, St. George's University, Grenada, West Indies.

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Methodology: A total of 65 blue land crabs were examined for the presence of Gram-negative aerobic bacteria in their intestines and hepatopancreas by culture. The isolated bacterial species were tested against 12 antibiotics using the disc diffusion method.
Results: Eighty-nine percent of crabs were culture positive. *Klebsiella pneumoniae* was the most common species (60%), followed by *Citrobacter freundii* (28%), *Enterobacter cloacae* (17%), *Salmonella* spp. (17%), and *Escherichia coli* (12%). *Vibrio* isolates included *V. alginolyticus* (8%), *V. parahaemolyticus* (5%), and *V. fluvialis* (3%). Antimicrobial susceptibility tests against 12 drugs showed susceptibility of all *K. pneumoniae, C. freundii, Enterobacter cloacae,* and *Escherichia coli* isolates to enrofloxacin, gentamicin, and imipenem. Resistance to ciprofloxacin, chloramphenicol, and trimethoprim-sulfamethoxazole was ≤ 7%. All *K. pneumoniae* isolates were resistant to ampicillin, and all *C. freundii* isolates were resistant to cephalothin. Resistance to tetracycline was highest (33%) in *E. cloacae,* and ≤ 13% in the other three major species. Susceptibility of *Salmonella* serotypes has been published, and no resistance was seen among any of the isolates.
Conclusion: This study showed that the blue land crabs of Grenada, commonly used as food, can serve as reservoirs of potential human pathogens, and may carry bacteria resistant to antimicrobial drugs used for treatment in human medicine.

Keywords: Klebsiella pneumonia; Salmonella; Enterobacter cloacae; ciprofloxacin; ciprofloxacin; tetracycline; Vibrio; St. George's.

ABBREVIATIONS

IACUC (Institutional Animal Care and Use Committee); TSB (Trypticase soy broth); TCBS (thiosulfate-citrate-bile salt-sucrose); MAC (MacConkey agar); CLSI (Clinical and Laboratory Standards Institute); FDA (Food and Drug Administration).

1. INTRODUCTION

Cardisoma guanhumi, the blue land crab, is found along the Gulf of Mexico and Caribbean Sea throughout the southeastern United States, Central America, the northern tip of South America, and parts of the Caribbean Islands which include Grenada [1]. These crustaceans are considered as the largest of Florida's semi-terrestrial crabs and are commonly exploited for food in Caribbean islands, including Grenada [1]. In Brazil, it is considered one of the most important crustacean species captured and commercialized [2]. In spite of the importance of crabs to humans, information on the bacteria that are generally associated with the crabs especially those associated with intestine and hepatopancreas of crabs is lacking. Because of the natural habitat of the blue land crabs, the preference of the crabs to inhabit low lying aquatic areas, their ability to move to and from multiple sites, and their dietary habits, researchers have hypothesized that these crabs may serve as reservoir for different bacterial species including those that are pathogenic to humans (such as *Salmonella* spp. and *Vibrio* spp.) as well as those that are potentially pathogenic to humans (such as *Escherichia coli*, *Klebsiella*, *Citrobacter*, and *Pseudomonas*). *Vibrio parahaemolyticus*, a causative agent of gastroenteritis in human have been isolated from crabs [3]. *Salmonella* spp, another major causative agent of gastroenteritis was recovered from the blue land crabs in Grenada [4].

Antibiotic resistance is a major problem in enteric bacteria such as *E. coli*, *Salmonella*, *Klebsiella*, and *Citrobacter*. Previous studies carried out in Grenada have shown that both wild and domesticated animal species including chicken [5-8], dogs [9], cats [10], pigs [11], cane toads [12] and green iguanas [13] may serve as reservoirs for antibiotic resistant bacteria and these animals can freely shed these antibiotic resistant organisms in the environment. Despite this, information on the antibiotic resistance profiles of the bacteria that are associated with the blue land crabs is lacking.

The findings of previous research on bacteria associated with different animal species as well as the association of crabs with humans and the environment have supported the notion that blue land crabs could be reservoir for antibiotic resistant bacteria that may be of zoonotic importance. In this context, the blue land crabs of Grenada constitute a suitable model to study the prevalence and antibiotic susceptibility profile of aerobic bacteria associated with crab. We

evaluated the prevalence of aerobic bacteria associated with the intestine and hepatopancreas of blue land crab and the susceptibility of the bacteria to a panel of antimicrobials that included some drugs used for the treatment of bacterial infections in the human and veterinary clinics in Grenada. This is the first report of isolation and antimicrobial susceptibilities of various Gram-negative aerobic bacteria cultured from the intestine and hepatopancreas of blue land crab in Grenada, West Indies.

2. MATERIALS AND METHODS

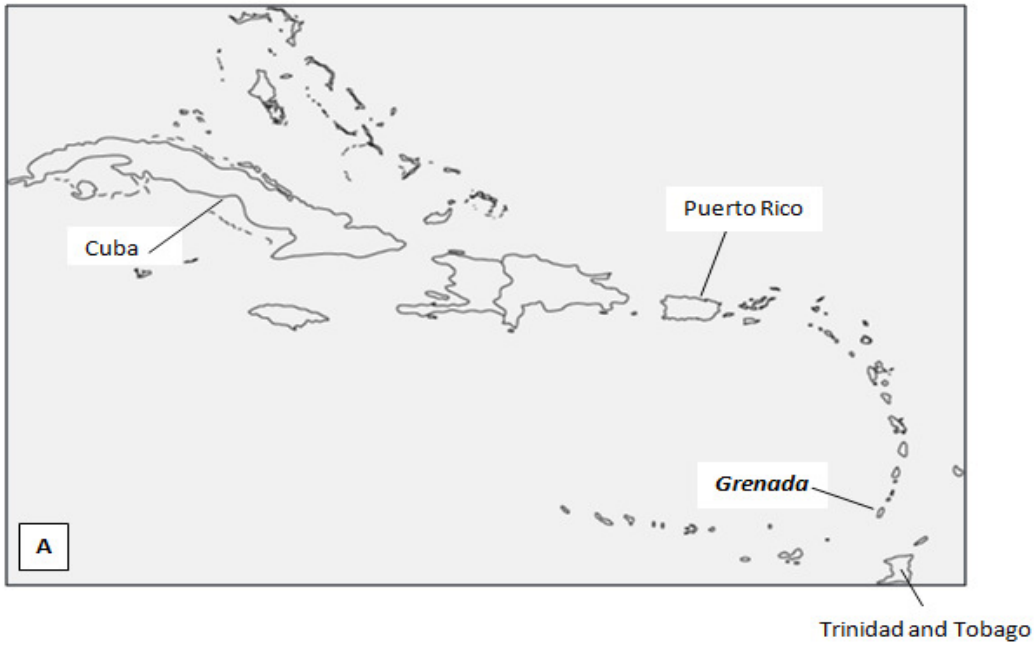
The study was approved by and conducted in accordance with the regulations of the St. George's University's Institutional Animal Care and Use Committee (IACUC-10009-R). A total of 65 crabs were collected during a three month period from November 2011 to February 2012 from six parishes of Grenada: 13 from St. George's, 12 from St. Andrew's, and 10 each from St. David's, St. John's, St. Mark's, and St. Patrick's parishes. A map representing the parishes where this study was conducted is shown in a previous publication [4] and presented in Fig. 1. All crabs obtained for the study were identified by parish of origin and date obtained.

The crabs were euthanized by placing them in chilled water (1°C) containing eugenol (0.125 ml/liter; Fisher Scientific, Pittsburg, PA) for 60 min. Homogenates of aseptically sampled intestines and hepatopancreas were prepared without addition of any liquid using a stomacher blender (Seward, Worthington, UK), and 0.1 ml of the suspension was inoculated into 10 ml of Trypticase soy broth (TSB; Remel, Lenexa, KS), a nonselective enrichment medium, and incubated at 37°C for 24 h. For the isolation of *Salmonella* and the identification of the different *Salmonella* serotypes, the further method used is described in a previous publication [4]. For the isolation of *Vibrio* and other bacterial species, after incubation, subcultures of the TSB culture were made by streak plating on thiosulfate-citrate-bile salt-sucrose agar (TCBS) (Sigma-Aldrich, St. Louis Mo. USA), which is a selective media for *Vibrio* spp. and on MacConkey agar (MAC) (Difco, BD, Sparks, MD), for the other bacterial species. The subcultures were then incubated at 37°C for 18-24 h. After incubation, colonies from the TCBS agar resembling *Vibrio* spp were subcultured the second time on TCBS and incubated at 37°C for 18 to 24 h. for isolation of pure colonies. Also all colonies from the MAC agar were subcultured the second time on MAC agar and incubated at 37°C for 18 to 24 h. All the colonies from the second TCBS and MAC agar plates were Gram stained and identified using their biochemical characteristics: oxidase test, fermentation of lactose, nitrate reduction, citrate utilization, urease and indole production, catalase production, reaction on triple sugar iron agar, and growth on TCBS agar plate. Colonies with characteristics that represents *Vibrio* and members of the family *Enterobacteriaceae* were inoculated into API 20E® (Analytical Profile Index; bioMérieux, Hazelwood, MO) strips and incubated at 37°C for 18 to 24 h. for further identification. API 20NE® (bioMérieux, Hazelwood, MO) strips were used for identification of *Aeromonas* and *Pseudomonas* spp.

Antimicrobial susceptibility testing was performed on all the major Gram-negative species using the disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [14] on Mueller-Hinton agar. The antibiotic discs used were amoxicillin–clavulanic acid, ampicillin, cephalothin, chloramphenicol, ciprofloxacin, enrofloxacin, gentamicin, imipenem, neomycin, streptomycin, tetracycline, and trimethoprim-sulfamethoxazole (BD, Franklin Lakes, NJ). The inhibition zone sizes for all drugs except neomycin were interpreted based on the CLSI guidelines [14]. For neomycin, the manufacturer's guidelines, as approved by the U.S. Food and Drug Administration (FDA), were used.

3. RESULTS

Of the 65 blue land crabs examined in this study, 58 (89%) showed the presence of one or more Gram-negative bacterial species in their hepatopancreas and/or intestine. In total, 22 bacterial species of 13 genera were cultured, 39 (60%) crabs were positive for *Klebsiella pneumoniae,* 18 (28%) for *Citrobacter freundii*, 11 (17%) for *Enterobacter cloacae*, 11 (17%) for *Salmonella* spp. and 8 (12%) for *E. coli* while the percentage of crabs positive for *Vibrio* and other bacterial species ranged from 2-9% (Table 1). Of the bacterial species cultured, 84 bacterial isolates were recovered from the intestine and 74 from the hepatopancreas which indicates that the bacterial species are more concentrated in the intestine compared to the hepatopancreas (Table 1).



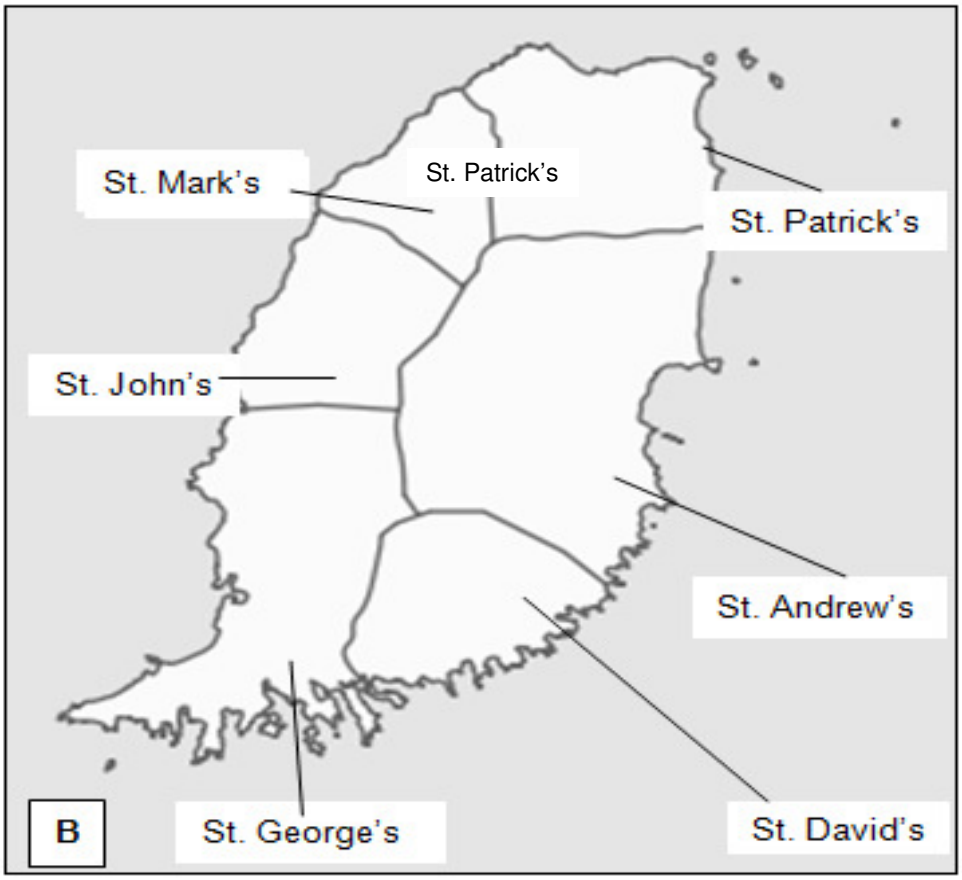


Fig. 1. (A) Location of Grenada; (B) sampling locations in Grenada

The antimicrobial susceptibility results presented are for only the predominant bacterial species, *K. pneumoniae, C. freundii*, *E. cloacae*, and *E. coli* recovered form the intestine of the positive crabs. The antimicrobial susceptibility result for *Salmonella* spp. has been published [4]. Our antimicrobial susceptibility tests revealed susceptibility of all the predominant bacterial species to enrofloxacin, gentamicin and imipenem. All *K. pneumoniae*, *E. cloacae*, and *E. coli* where also susceptible to ciprofloxacin; *K. pneumoniae* and *E. coli* to neomycin; *C. freundi*, *E. cloacae* and *E. coli* to chloramphenicol; *E. cloacae* and *E. coli* to streptomycin and trimethoprim-sulfamethoxazole; and *E. coli* to tetracycline. Resistance was observed among all *K. pneumoniae* and *C. freundii* to ampicillin and cephalothin, respectively. The antibiotic susceptibility profiles of the four predominant bacterial species recovered from the positive crabs are presented in Table 2. Some of the predominant bacterial species showed resistance to one of the antibiotics tested (single antibiotic resistance) while some showed resistance to two or more (multiple antibiotic resistance): Overall, 21 (84%) of *K. pneumoniae* showed resistance to single antibiotic while four (16%) to multiple antibiotics; two (13%) of *C. freundii* to single antibiotic, while 13 (87%) to multiple antibiotics; one (17%) of *E. cloacae* to single antibiotic while four (67%) to multiple antibiotics; and one (17%) of *E. coli* showed resistance to single antibiotics while three (50%) to multiple antibiotics. The most common resistance seen among the multiple resistant *C. freundii*, *E. cloacae*, and *E. coli* was for amoxicillin–clavulanic acid, ampicillin and cephalothin.

4. DISCUSSION

Several Gram-negative aerobic bacterial species were recovered from the 58 positive crabs out of the 65 crabs that were tested during the study period from the six parishes of Grenada. *K. pneumoniae, C. freundii*, *Salmonella* spp., *E. cloacae* and *E. coli* were the most predominant species (30%, 18%, 13%, 7% and 7%, respectively) recovered from intestine of the positive crabs (Table 1). *Klebsiella* spp. are ubiquitous in nature and thrives in tropical and subtropical regions. The high prevalence of *K. pneumoniae* observed in this study may be due to the ubiquitous nature of *Klebsiella*. This organism has two common habitats, one being the environment, where they are found in surface water, sewage, and soil and on plants [15-17] and the other being the mucosal surfaces of mammals such as humans, horses, or swine, which they colonize. In humans, *Klebsiella* spp are mainly known for causing respiratory and urinary tract infections, and diarrhea [18]. Previous study in Canada by Rennie et al. [19] revealed the association of *K. pneumoniae* in an acute gastro-enteritis linked to ingestion of turkey contaminated with a rare serotype of *K. pneumoniae*, capsular type K-15, which produces an LT-like enterotoxin. *Klebsiella* spp. have also been identified as important common pathogens for nosocomial pneumonia (7 to 14% of all cases), septicaemia (4 to 15%), urinary tract infection (UTIs; 6 to 17%), wound infections (2 to 4%), intensive care unit (ICU) infections (4 to 17%), and neonatal septicaemias (3 to 20%) [20]. *Klebsiella oxytoca*, another *Klebsiella* spp recovered in this study, can also cause community-acquired meningitis and brain abscesses and its host range include human, animals such as ringtail possums, gliders, and bats [20].

C. freundii, the second most predominant species recovered in this study can be found throughout the environment including the soil and water, and it is a normal constituent of the intestinal tracts of animals and humans [21]. As an opportunistic pathogen, *C. freundii* is known to be the cause of a variety of nosocomial infections of the respiratory tract, urinary tract, and blood in humans, *C. freundii* represents approximately 29% of all opportunistic infections reported [22,23]. In a small town in northwest Germany, there has been a report of a Shiga-toxin producing strain of *C. freundii* causing an outbreak of gastro-enteritis followed by hemolytic uremic syndrome (HUS) in a nursery school [24]. The outbreak was traced to butter mixed with parsley, colloquially known as “Green Butter”. Neonates and immunocompromised, elderly or debilitated patients are at increased risk of infection caused by *Citrobacter* spp. [21,25]. *Enterobacter cloacae*, one of the most predominant bacterium recovered in our study are widely encountered in nature, but they can act as pathogens, particularly in nosocomial bloodstream infections in the last decade, little is known about their virulence-associated properties [26].

The prevalence, serovars, and susceptibility of the *Salmonella* isolates from this present study have been published [4], overall, the individual animal prevalence of *Salmonella* based on isolation was 17% (11 of 65), and all infected crabs were from three of the six sampled

locations (St. George's, St. Patrick's, and St. David's). Isolates were identified by serotyping as *Salmonella enterica* serovars Saintpaul (n = 6), Montevideo (n = 4), and Newport (n = 1). The intestines of all 11 infected crabs were positive for *Salmonella*, but only 7 of 11 hepatopancreas samples were positive for *Salmonella*, and these isolates were the same serovar as isolated from the matching intestine. These three *Salmonella* serovars are known to cause human illness in many countries, and in the Caribbean *Salmonella* Saintpaul has been frequently isolated from humans. No resistance was seen among any of the *Salmonella* isolates.

Our study showed that *E. coli* was also predominant in the intestine of the land crabs. Sylvester, et al. [13] found that 40% (25 out of the 62) of green iguanas were positive for *E. coli*. Many other studies in Grenada have also shown high prevalence of *E. coli* in different animal species: in 2011, 102 *E. coli* isolates were recovered from 113 fecal samples of pigs [11], also 55 *E. coli* isolates were recovered from 180 egg samples (90 yolks and 90 shell membrane) [6]; in 2007 and 2008, 203 and 183 *E. coli* isolates were recovered from 207 and 197 samples of caeca from chickens, respectively [7,8]. Generally, *E. coli* is considered as a commensal inhabitant of gastrointestinal tract of humans and animals, although some strains (example *E. coli* O157:H7) are known to cause serious morbidity and mortality occasionally linked to food poisoning. In 2012, an outbreak of gastro-enteritis in Plymouth, England was associated with crab meat contaminated with *E. coli* 0157:H7 [27]. This was the first recorded incident that *E. coli* O157:H7 was associated with the consumption of crabmeat. This present study was not designed to determine the occurrence of *E. coli* O157:H7 in crabs. However, the study of Sylvester, et al. [13] did not show the presence of *E. coli* O157:H7 in green iguanas. *E. coli* is not considered to be part of the normal flora of marine crabs, due to the fact that *E. coli* is rapidly eliminated from sea water [28]. In the crab processing industry in the United States, the detection of coliforms including *E. coli*, is an indication of unsanitary handling of crabs and crab meat [29]. Although there is no documentation to substantiate whether *E. coli* is considered a commensal in the blue land crab, a study was conducted on the mud crab (*Scylla serrate*) of Malaysia, which inhabits a similar estuarine environment and has similar dietary habits as the blue land crab [30]. In that study, *E. coli* was considered to be cultured as a result of fecal contamination. Due to the similarity of habitat of the mud crab and the blue land crab, it could be extrapolated that *E. coli* was cultured in the blue land crab due to environmental factors, and as in the case of *Salmonella* spp. [4].

Our study revealed the presence of *V. parahemolyticus* and other related *Vibrio* spp (*V. alginolyticus* and *V. fluvialis*) in the hepatopancreas and intestine of land crabs (Table 1). Although we observed a low prevalence of *Vibrio* spp compared to *K. pneumoniae, C. freundii*, *Salmonella* spp., *E. cloacae* and *E. coli*, their presence in crabs is of public health significance due to the pathogenic characteristics, disease burden, morbidity, and mortality associated with *Vibrio* spp. In a study on blue crabs, *Callinectes sapidus*, in USA, a high prevalence of *V. parahemolyticus* (21%) was reported [3]. *V. parahemolyticus* has been reported as a public health problem in the commercial preparation of crab meat [31,32]. In 1968, Moribund blue crabs was reported to contain *V. parahaemolyticus* [33]. Many outbreaks of gastroenteritis associated with the consumption of crabs contaminated with *V. parahaemolyticus* have been reported [34,35]. The most common clinical syndrome of exposure to *V. parahemolyticus* is gastro-enteritis [36]. *V. parahemolyticus* was first implicated as a cause of gastro-enteritis in Japan in 1950 [37]. It is now known as the leading cause of seafood-associated bacterial gastro-enteritis worldwide [38,39]. This organism is found throughout the coastal regions of the world, including many areas of the USA. It can be cultured from several sources: marine fish, shellfish, mud, sediment, and water samples from bays and estuaries.

We found a low prevalence (1 – 5%) of other bacterial genera in the hepatopancreas and/or intestine of crabs, these including *Aeromonas* spp., *Kluyvera* spp., *Morganella* spp., *Pantoea* spp., *Pseudomonas* spp., *Raoultella* spp., and *Serratia* spp. (Table 1). Some of these bacterial isolates including *Aeromonas* spp and *Pseudomonas* spp have been previously isolated from blue crabs [3].

In this study, we determined the antimicrobial profiles for the four predominant bacterial species, that is, *K. pneumoniae*, *C. freundii*, *E. cloacae* and *E. coli* (Table 2). All *K. pneumoniae* isolates in the present study showed resistance to ampicillin (100%).

Table 1. Gram-negative bacterial isolates cultured from the hepatopancreas and intestine of positive blue land crabs in Grenada

S/N	Bacterial isolate	Positive organs for bacterial isolate		Positive crabs for bacterial isolates** (n = 65) # (%)
		Hepatopancreas # (%)	Intestine # (%)	
1	*Aeromonas* spp.	2 (3)	2 (2)	4 (6)
2	*Citrobacter braakii*	0 (0)	1 (1)	1 (2)
3	*Citrobacter freundii*	8 (11)	15 (18)	18 (28)
4	*Citrobacter youngae*	2 (3)	2 (2)	3 (5)
5	*Enterobacter aerogenes*	2 (3)	1 (1)	1 (2)
6	*Enterobacter asburiae*	0 (0)	1 (1)	1 (2)
7	*Enterobacter cancerogenus*	0 (0)	1 (1)	1 (2)
8	*Enterobacter cloacae*	6 (8)	6 (7)	11 (17)
9	*Enterobacter sakazakii*	1 (1)	0 (0)	1 (2)
10	*Escherichia coli*	5 (7)	6 (7)	8 (12)
11	*Klebsiella oxytoca*	4 (5)	2 (2)	6 (9)
12	*Klebsiella pneumoniae*	24 (32)	25 (30)	39 (60)
13	*Kluyvera* spp.	0 (0)	1 (1)	1 (2)
14	*Morganella morganii*	0 (0)	1 (1)	1 (2)
15	*Pantoea* spp.	1 (1)	3 (4)	4 (6)
16	*Pseudomonas* spp.	4 (5)	0 (0)	4 (6)
17	*Raoultella terrigena*	2 (3)	1 (1)	1 (2)
18	*Salmonella* spp.	7 (9)	11 (13)	11 (17)
19	*Serratia odorifera*	0 (0)	1 (1)	1 (2)
20	*Vibrio alginolyticus*	2 (3)	3 (4)	5 (8)
21	*Vibrio fluvialis*	2 (3)	0 (0)	2 (3)
22	*Vibrio parahaemolyticus*	2 (3)	1 (1)	3 (5)
	Total #	**74**	**84**	

***Hepatopancreas and/or intestine, S/N: Serial Number, #: Number, %: Percentage*

Table 2. Antimicrobial susceptibility profiles of the predominant bacterial isolates cultured from the intestine of blue land crabs in Grenada

Bacterial isolate	***Klebsiella pneumoniae* (n = 25)**		***Citrobacter freundii* (n = 15)**		***Enterobacter cloacae* (n = 6)**		***Escherichia coli* (n = 6)**	
Antimicrobial (Disc conc. [a] (µg))	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**
	%							
Amoxicillin–clavulanic acid (20, 10)	4	96	73	27	50	50	50	50
Ampicillin (10)	100	0	80	20	50	50	33	67
Cephalothin (30)	4	96	100	0	67	33	67	33
Chloramphenicol (30)	4	96	0	100	0	100	0	100
Ciprofloxacin (5)	0	100	7	93	0	100	0	100
Enrofloxacin (5)	0	100	0	100	0	100	0	100
Gentamicin (10)	0	100	0	100	0	100	0	100
Imipenem (10)	0	100	0	100	0	100	0	100
Neomycin[b] (30)	0	100	33	67	50	50	0	100
Streptomycin (10)	4	96	27	73	0	100	0	100
Tetracycline (30)	8	92	13	87	33	67	0	100
Trimethoprim-sulfamethoxazole (1.25, 23.75)	4	96	7	93	0	100	0	100

[a] *Susceptible (S) or Resistant (R) according to CLSI guideline for all drugs except neomycin*
[b] *For neomycine, FDA-approved manufacturer's (BD) guideline were used.*

This is not surprising because *K. pneumoniae* is intrinsically resistant to aminopenicillins (ampicillin), carboxypenicillins (carbenicillin) and other β-lactam antibiotics [40]. Some strains of *K. pneumoniae* that have not acquired any resistance determinants still are able to produce a chromosomal penicillinase which enable them to resist killing by antibiotics such as ampicillin and carbenicillin [40]. Our *C. freundii* isolates showed highest rate of resistance to four drugs including cephalothin (100%), ampicillin (80%), neomycin (33%) and streptomycin (27%). The resistance of all *C. freundii* to cephalothin in this present study is similar to the findings of Li et al., [41]. In this present study, only *C. freundii* (7%) showed resistance to ciprofloxacin (Table 2). The resistance rate for ciprofloxacin in our study was lower compared to 66.7% observed from clinical isolates in India [42].

5. CONCLUSION

Our study generated a baseline data indicating that the blue land crabs in Grenada commonly exploited for food can serve as reservoir for potential human pathogens, and may carry bacteria resistant to antimicrobial drugs used for treatment in human medicine.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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