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Multiple antibiotic resistances of enteric bacteria isolated from recreational coastal waters and oysters of the Caribbean Sea

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Abstract - This study investigated the antibiotic resistance of enteric bacteria and their association with environmental factors in a coastal area of the Caribbean Sea. Seawater and oyster samples were collected during rainy and dry seasons. Faecal indicators of seawater fulfilled international standards, except for enterococci during dry season, while in oysters were above 800 MPN/g tissue. Different cultural methods were used to isolate enteric bacteria, further identified by biochemical tests for species of the genera *Escherichia*, *Providencia*, *Kluyvera*, *Citrobacter*, *Morganella*, *Klebsiella* and *Enterococcus*. A total of 21 isolates presented multiple antibiotic resistances at least to five antibiotics, with higher resistance for *Enterococcus durans* against 20 antibiotics tested (20/20), followed by *Escherichia coli* (9/20). Enteric bacteria isolated from any sample source during the rainy season presented the highest antimicrobial resistance to penicillins and cephalosporins. This is the first report of multiple antibiotic resistances in enteric environmental isolates at the Caribbean Sea. This coastal environment might serve as a reservoir of antibiotic resistant bacteria and might represent public health risks associated with the use of recreational waters and the consumption of raw seafood.

Key words: multiple antibiotic resistances; enteric bacteria; seawater; oysters; public health risks.

INTRODUCTION

Antimicrobial resistance in bacteria associated with food and aquatic environments has become an emerging global health and economical concern (Lima-Bittencourt *et al.*, 2007; Watkinson *et al.*, 2007; Van *et al.*, 2008). Extensive and intensive use and misuse of antibiotics in medication, veterinary, agriculture and aquaculture have resulted in new selective pressures on environmental antibiotic resistant bacteria to be widespread in the environment (Reinthaler *et al.*, 2003; Kummerer, 2004; Constanzo *et al.*, 2005).

Antibiotic resistance in enterobacteria and enterococci, partially primary or opportunistic pathogens, has increased dramatically in aquatic environments (Arvanitidou *et al.*, 2001; Schwartz *et al.*, 2003; Dang *et al.*, 2008a, 2008b; Wang *et al.*, 2008). The majority of antibiotic resistance studies have focused on pathogenic bacterial populations, but it is known that commensal bacteria are also common reservoirs of antibiotic resistance genes (Salyers *et al.*, 2004). Antibiotic resistant strains can reach the environment through manure of animals as well as through human excretions (Reinthaler *et al.*, 2003). The potential for this resistance to be transferred to native populations or other pathogenic species is largely unknown (Watkinson *et al.*, 2007). Therefore, it is considered important to study the antimicrobial resistance in pathogenic as well as commensal bacteria in aquatic environments (Biyela *et al.*, 2004).

Worldwide studies have identified the aquatic ecosystems as possible reservoirs of bacterial antibiotic resistance (Gõni-Urriza *et al.*, 2000; Biyela *et al.*, 2004; Kümmerer, 2004; Dang *et al.*, 2006a, 2006b, 2007; Lima-Bittencourt *et al.*, 2007; Watkinson *et al.*, 2007; Dang *et al.*, 2008a, 2008b; de Oliveira and Pinhata, 2008; Wang *et al.*, 2008). High incidence of resistant bacteria in response to antibiotic usage have also been reported in coastal areas, considered as a serious biotic contamination and a means for the spread and evolution of resistance genes and their vectors (Young, 1993; Herwig *et al.*, 1997; Dang *et al.*, 2008a, 2008b; Wang *et al.*, 2008). However, there are scarce reports from the Caribbean Sea. Our previous studies indicated the presence of potentially pathogenic bacteria with multiple antibiotic resistances at the northwestern coast of Venezuela (Fernández-Delgado *et al.*, 2007, 2009). In the current work, we determined the antibiotic resistance pattern of enteric bacteria isolated from an important touristic and shellfish-growing marine area, in order to gain a basic understanding of the occurrence of resistant bacteria and the potential influence of environmental factors.

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MATERIALS AND METHODS

Sampling site and sample collection. Seawater samples were collected at three sites in the area of Chichiriviche at the northwestern coast of Venezuela, during the rainy (December 2004) and dry (May 2005) seasons. Sampling site 1 (Lat. 10°55'30"N, Long. 68°17'11"W) was at the mouth of a creek called Los Pozones near the touristic town of Chichiriviche. Site 2 (10°54'23"N, 68°18'10"W) was at Cueva de la Virgen, a rocky shoreline cave-like formation frequently visited by tourists. Site 3 (10°18'17"N, 68°05'01"W) was above a sunken ship near Chichiriviche at Cuare Gulf. Duplicate seawater samples were taken 10 cm below the surface and placed in sterilized glass bottles. The seawater in situ values of pH (pHep1, Hanna Instruments, Woonsocket, RI, USA), salinity (RHS-10ATC refractometer, Westover Scientific, Mill Creek, WA, USA), temperature and dissolved oxygen (OXDP-02 oxygen meter, VWR International, Inc., West Chester, PA, USA) were measured. Live specimens of *Isognomon alatus* and *Crassostrea rhizophorae* oysters, were collected from the surfaces of the sunken ship (site 3) and from mangrove roots of Cuare Gulf, respectively. Two samples of 60 individuals of each species were washed with sterile distilled water and placed in sterilized glass bottles. These marine samples were kept refrigerated until their transportation to the laboratory, where they were processed within eight hours of sampling.

Bacteriological assessment of seawater and oyster tissue. The total and faecal coliforms and enterococci in seawater and oyster tissue homogenates were estimated as the Most Probable Number (MPN) in aliquots of 0.01, 0.1, 1 and 10 ml, using the Multiple Tube Fermentation (MTF) technique (APHA, 1995). The oyster shells were washed thoroughly with sterile water then opened and the tissues removed; 10 g of tissue was homogenized in 90 ml PBS buffer (0.32 mol l^{-1}) at pH 7.2, by duplicate. The initial dilutions for oyster tissue analyses were 1:10 and 1:100 for rainy and dry seasons, respectively. For the determinations of total coliforms, Lauryl Sulfate (Merck, Darmstadt, Germany) and Bright Green Bile (BRILA, Merck) were used for incubations at 35 °C for 24-48 h, and the presence of faecal coliforms was confirmed in *Escherichia coli* medium (EC, Merck) at 44.5 °C for 24-48 h. For enterococci, the samples were cultured in Dextrose Azide broth (Merck) with confirmation in Ethil Violete Azide broth (EVA, Merck,) at 35 °C for 24-48 h (APHA, 1995).

Bacterial isolation and biochemical identification. Aliquots of 50 µl were taken from the dilutions of seawater and oyster samples which were positive for total and faecal coliforms and enterococci, and were inoculated in MacConkey (HIMEDIA, Mumbai, India) and mEnterococcus (Difco, Detroit, MI, USA) media, respectively. ENDO (HIMEDIA) and Bile Esculine Azide Agar (HIMEDIA) were also used for isolation purposes. The cultures were grown under aerobic conditi ns at 37 °C for 24-48 h.

Microbiological tests were performed according to standard methods on representative isolates. The biochemical characteristics of these isolates were determined with API ID 32E and API ID 32STREPT systems (BioMérieux, Lyon, France). A dendrogram based on the biochemical reactions of enterobacteria isolates was produced with the computer package MINITAB 14.2 (www.minitab.com) by using the single linkage of cluster analysis applied to similarities based on the Manhattan distance.

Antibiotic resistance. A total of 21 enteric isolates were tested for their resistance patterns against 20 different therapeutic antibiotics, commonly prescribed for clinical infections in Venezuela. The antibiotic resistance was evaluated following the standard agar disc diffusion technique (Bauer *et al.*, 1966). Möeller-Hinton Agar (Becton Dickinson, Sparks, MD, USA) plates were incubated at 37 °C for 24-48 h. Interpretation of results was carried out considering diameters of inhibition halos of less than 5 mm as resistant, between 5 and 12 mm as intermediate, and larger than 12 mm as sensitive.

RESULTS

Environmental parameters and bacteriological assessment

During the sampling periods, the physicochemical parameters of seawater were pH from 7.9 to 8.9, temperature from 29.5 to 39.9 °C, salinity from 9 to 34‰, and dissolved oxygen from 4.2 to 6.4 mg O_2 l^{-1}. The major difference at this coastal environment was seawater salinity (9-11‰) during rainy season, which was lower than at the dry season, possibly due to drainage of rainwater at sampling time. The estimates for total and faecal coliforms were below 50 MPN/100 ml, whereas enterococci values were at a maximum of 40 MPN/100 ml in seawater samples for both rainy and dry seasons. Faecal indicators of seawater fulfilled local and international standards, except for enterococci during dry season at sampling site 1. In both oyster species, all the indicators were above 16000 MPN/g of tissue, with lower values for enterococci in *I. alatus* (200-800 MPN/g of tissue) (Table 1).

Identification of environmental isolates

Twenty one representative enteric isolates from both seasonal periods were selected from seawater (Col DIC 1, Col DIC 3, Col DIC 4, Col DIC 10, Col DIC 11, Col MAY 17, ENT DIC AB and ENT MAY 2), from *I. alatus* (Col DIC

TABLE 1 - Most Probable Number of faecal indicators in seawater and oysters from the Caribbean Sea during rainy and dry seasons

Season	Total Coliforms					Faecal Coliforms					Enterococci				
	Seawater			Oyster species		Seawater			Oyster species		Seawater			Oyster species	
	Site 1	Site 2	Site 3	*I. alatus*	*C. rhizophorae*	Site 1	Site 2	Site 3	*I. alatus*	*C. rhizophorae*	Site 1	Site 2	Site 3	*I. alatus*	*C. rhizophorae*
Rainy	50	22	13	≥ 16000	≥ 16000	14	17	4	≥ 16000	≥ 16000	2	13	4	800	≥ 16000
Dry	40	33	2	≥ 160000	≥ 160000	20	4	<2	≥ 160000	≥ 160000	40	17	<2	200	≥ 160000

13, Col DIC 18, Col MAY 3 and Col MAY 18) and from *C. rhizophorae* (Col DIC 15, Col DIC 21, Col DIC 27, Col MAY 1, Col MAY 14, Col MAY 19, ENT MAY 5, ENT MAY 6 and ENT MAY 7). Several enterobacterial isolates were identified as *Providencia rettgeri, Kluyvera ascorbata, Citrobacter freundii, Morganella morganii, Klebsiella pneumoniae* and *Escherichia coli* (Fig. 1), the latter being the most commonly isolated from these marine samples. The dendrogram shows the biochemical relationships between the isolates and some reference microorganisms used at the API ID 32E. The isolates Col DIC 1, Col DIC 15, Col DIC 18, Col DIC 21, Col MAY 1, Col MAY 3, Col MAY 18 and Col MAY 19 form a phenon cluster with *E. coli* (76.92% similarity); Col DIC 4 with *K. ascorbata* (96.15% similarity); Col DIC 10 with *C. freundii* (96.15% similarity); Col DIC 13 and Col DIC 27 with *K. pneumoniae* (96.15% similarity); Col DIC 11 with *M. morganii* (100% similarity); and finally Col DIC 3 with *P. rettgeri* (92.31% similarity). For comparative purposes, *Vibrio cholerae* was included as an outgroup to the Enterobacteriaceae family.

The *Enterococcus* isolates, presumptively identified as *E. durans* (ENT DIC AB), *E. faecium* (ENT MAY 2 and ENT MAY 6), *E. faecalis* (ENT MAY 5) and *E. hirae* (ENT MAY 7) with similarities between 61.4-99.9%, showed biochemical features common to the genus (Devriese *et al.*, 1992).

Antibiotic resistance

The antibiotic resistance patterns of the enteric bacterial isolates evaluated are summarized in Table 2. Multiple antimicrobial resistances were observed between the isolates, showing resistance to at least five antibiotics.

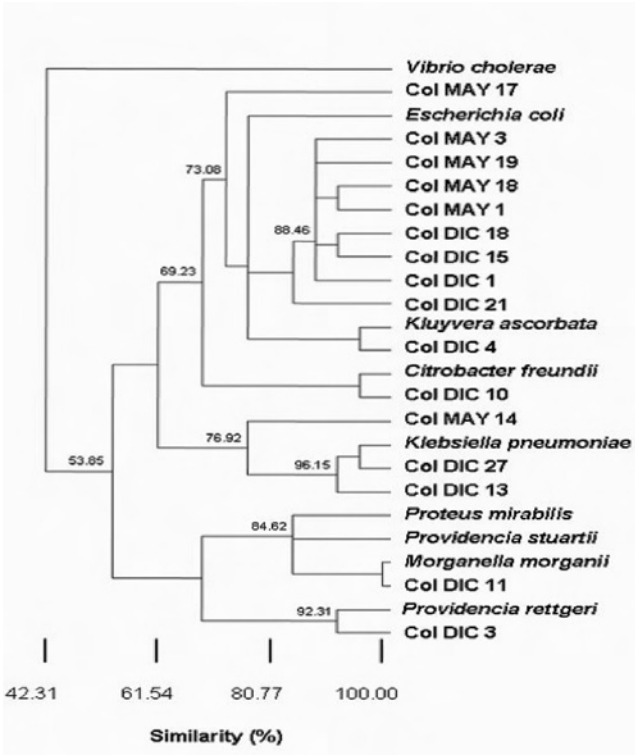

FIG. 1 - Dendrogram based on API ID 32E biochemical reactions showing the levels of similarity between all the enterobacterial isolates and reference strains from the ID32 E System of Enterobacteriaceae. The graph shows similarity percentages by single-linkage method with Manhattan distances.

The highest incidence of bacterial resistance was recorded for penicillin, followed by ampicillin/sulbactam and carbenicillin. All of the isolates were resistant to penicillin, except *M. morganii* and *C. freundii*. The enterococci were additionally resistant to polymyxin B. All enterobacterial and enterococci isolates were sensitive to gentamicin, lomefloxacin and trimethoprim-sulfamethoxazole, except *E. durans*, which was resistant to all the antibiotics tested. The enterobacterial isolates presented susceptibility to ceftazidime, cefixime, gentamicin, kanamicin, lomefloxacin, netromicin, trimethoprim-sulfamethoxazole and tobramicin, whereas the enterococci were sensitive to ampicillin, ampicillin/sulbactam, cefamandole, carbenicillin, gentamicin and lomefloxacin (Table 2).

Our results show that resistance levels were particularly high among *E. coli* isolates compared with the other enterobacteria. *E. durans* was the only one resistant to gentamicin, lomefloxacin and trimethoprim-sulfamethoxazole. Among the antimicrobial agents tested, the highest resistance rates were found in the penicillins and cephalosporins families for enterobacteria, and aminoglycosides and polymyxin B for enterococci (Table 2).

Enteric bacteria displaying multiple antimicrobial resistances were predominant in the rainy season, with highest resistance patterns to ampicillin/sulbactam and carbenicillin in the penicillin family, followed by cefazolin and cefoxitin in the cephalosporins family. All the isolates collected during the dry season were resistant to penicillin, with lower resistance profiles than the isolates collected during the rainy season for the same antibiotics assayed (Fig. 2A). Enteric bacteria isolated from seawater presented the highest multiple antimicrobial resistances, except to penicillin, followed by bacteria isolated from *C. rhizophorae* and *I. alatus*, respectively. At least one seawater isolate showed resistance to all of the antimicrobial agents tested. The oyster isolates displayed sensitivity to netromicin, amikacin, gentamicin, polymyxin B and trimethoprim-sulfamethoxazole (Fig. 2B).

DISCUSSION

Our results reveal the presence of enteric bacteria in seawater and in oyster tissue, suggesting exposure to faecal contamination in these aquatic environments. The presence of enteric bacteria such as *E. coli*, *K. pneumoniae*, *P. rettgeri, M. morganii, C. freundii* and *Enterococcus* spp. may be attributed to the drainage of contaminated water bodies, which increases during rainy periods and modify the physicochemical conditions of these coastal areas. These isolates presented multiple antimicrobial resistances to several penicillins, cephalosporins, aminoglycosides and polymyxin B, with higher resistance rates for *E. coli* among the enterobacterial isolates and *E. durans* among the enterococci. Considering all the antimicrobial agents assayed, the quinolones and sulfonamides appeared to be the most effective for these bacterial groups.

Other studies have reported the isolation of *E. coli* with multiple antibiotic resistances from seawater and oysters (Cardonha *et al.*, 2004; Kumar *et al.*, 2005; Watkinson *et al.*, 2007; Dang *et al.*, 2008a, 2008b; Wang *et al.*, 2008), and some evidence of the presence of *Citrobacter* spp., *Kluyvera* spp., *Morganella* spp. and *Providencia* spp. carrying antibiotic resistance has been reported recently in natural and marine environments (Lima-Bittencourt *et al.*, 2007;

TABLE 2 - Antibiotic resistance of enteric isolates from seawater and oysters during rainy and dry seasons

Family	Antibiotics	Number of isolates with antibiotic resistance									
		Escherichia coli (n = 9)	*Klebsiella pneumoneae* (n = 3)	*Providencia rettgeri* (n = 1)	*Kluyvera ascorbata* (n = 1)	*Citrobacter freundii* (n = 1)	*Morganella morganii* (n = 1)	*Enterococcus durans* (n = 1)	*Enterococcus faecium* (n = 2)	*Enterococcus faecalis* (n = 1)	*Enterococcus hirae* (n = 1)
Penicillins	PEN (2 U)	9	3	1	1	0	0	1	2	1	1
	AMP (10 µg)	4	3	1	1	0	0	1	0	0	0
	AMP/S (10 µg)	4	2	1	1	1	1	1	0	0	0
	NET (30 µg)	0	0	0	0	0	0	1	1	0	0
	CARB (100 µg)	4	2	1	1	1	1	1	0	0	0
Cephalosporins	CFX (30 µg)	4	2	1	1	0	0	1	0	1	0
	CMD (30 µg)	1	1	0	0	0	0	1	0	0	0
	CAZ (30 µg)	0	0	0	0	0	0	1	2	0	0
	KZ (30 µg)	4	0	1	1	1	0	1	1	0	1
	CRO (30 µg)	0	1	0	0	1	1	1	0	0	1
	CFIX (5 µg)	0	0	0	0	0	0	1	1	1	0
	RAD (30 µg)	2	0	1	1	1	0	ND	2	1	1
Aminoglycosides	AMK (30 µg)	0	0	0	0	0	1	1	1	0	0
	GEN (10 µg)	0	0	0	0	0	0	1	0	0	0
	KM (30 µg)	0	0	0	0	0	0	1	2	1	0
	NEO (5 µg)	1	0	0	0	0	0	1	1	0	0
	TOB (10 µg)	0	0	0	0	0	0	1	2	1	0
Polymyxin	POL (300 µg)	0	0	1	1	0	1	1	2	1	1
Sulfonamide	SXT (25 µg)	0	0	0	0	0	0	1	0	0	0
Quinolone	LFLX (10 µg)	0	0	0	0	0	0	1	0	0	0

PEN: penicillin G, AMP: ampicillin, AMP/S: ampicillin/sulbactam, NET: netromicin, CARB: carbenicillin, CFX: cefoxitin, CMD: cefamandole, CAZ: ceftazidime, KZ: cefazolin, CRO: ceftriaxone, CFIX: cefixime, RAD: cephradine, AMK: amikacin, GEN: gentamicin, KM: kanamicin, NEO: neomycin, TOB: tobramicin, POL: polymyxin B, STX: trimethoprim-sulfamethoxazole, LFLX: lomefloxacin. Concentrations are given per ml.

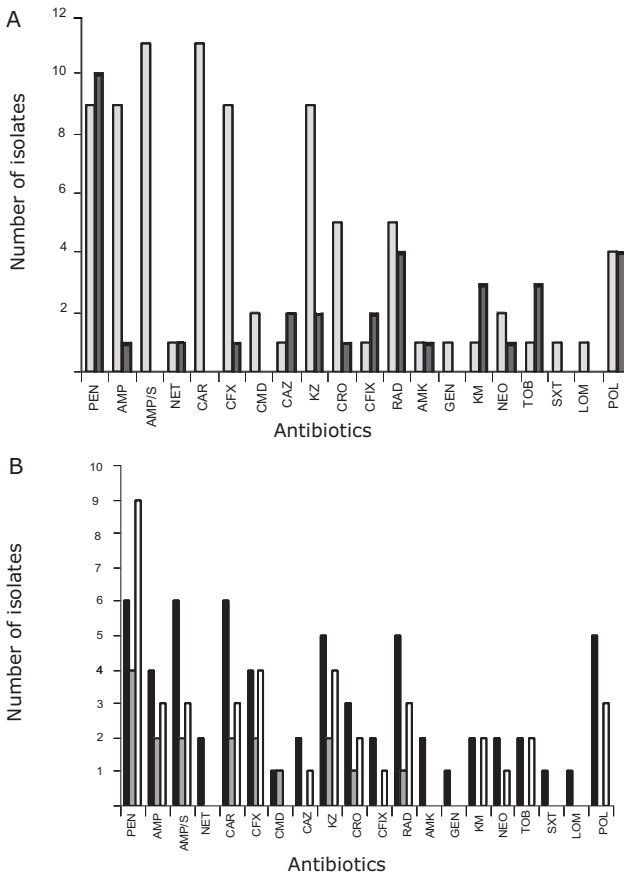

FIG. 2 - Distribution of enteric bacteria resistant to therapeutic antibiotics (see Table 2). A: Resistant by seasons. □: rainy season (n = 11), ■: dry season (n = 10). B: Resistant by samples. ■: seawater (n = 8), ▒: *Isognomon alatus* (n = 4), □: *Crassostrea rhizophorae* (n = 9).

Dang *et al.*, 2008a; Wang *et al.*, 2008). Our results highlight a significant resistance to the penicillin family among all the enteric isolates. Many coliforms are considered intrinsically resistant to these drugs (Goñi-Urriza *et al.*, 2000). Here we report *E. coli* isolates with 100% resistance to penicillin and 44.4% resistance to ampicillin, ampicillin/sulbactam, carbenicillin and netromicin. Other studies also noted that ampicillin-resistance was common in enteric bacteria isolated from environmental sources (Salyers *et al.*, 2004; Lima-Bittencourt *et al.*, 2007; Wang *et al.*, 2008).

Enterococcus spp. from clinical and environmental origins have been found with multiple antimicrobial resistances (Bustamante *et al.*, 2003; de Oliveira and Pinhata, 2008). The great majority of enterococci are involved in nosocomial infectious such as urinary tract infections, bacteremia, endocarditis, and others (Bustamante *et al.*, 2003). In the environment they are commonly isolated from sewage, aquatic habitats, agricultural run-off and animal sources, which indicates their ability to enter the human food chain. They are naturally resistant to cephalosporins and aminoglycosides, and intermediate sensitive to several penicillins (Lukášová and Šustácková, 2003). However, all our isolates were resistant to penicillin, with variable resistances to cephalosporins and aminoglycosides, possibly indicating an acquired resistance to penicillin. Although this genus is notorious for high-level gentamicin resistance, our isolates were sensitive with the exception of *E. durans*. Previous reports have shown that high-level resistance to gentamicin is rarely detected among enterococci isolated from the aquatic environment (Rice *et al.*, 1995; Arvanitidou *et al.*, 2001). Resistance to other aminoglycosides (i.e. kanamycin) has been reported for enterococci from Mediterranean coastal areas (Arvanitidou *et al.*, 2001) as it has been found in this research. Enterococci are known to acquire antibiotic resistance with relative ease and be able to spread these resistance genes to other bacteria (Kühn *et al.*, 2000). In Venezuela, like in other Latin American countries, until recent years medical prescription was not required to buy therapeutic antibiotics (OPS, 2004), and they are still very easily prescribed by doctors. An extensive use of antibiotics could lead to the dissemination of resistant bacteria in aquatic environments of the Caribbean Sea.

Multiple antimicrobial resistant isolates were found predominantly in samples collected during rainy season and in samples from seawater and *C. rhizophorae* (Fig. 2A and 2B). The variations in the patterns of resistance among the isolates during the rainy and dry seasons probably reflect changes in the composition of the bacterial populations sampled (Lima-Bittencourt *et al.*, 2007). During the rainy season, the surface runoff might introduce resistant strains into the marine ecosystem. The lower antibiotic resistant enteric bacteria populations in dry season could be due to a lower exogenous input from the terrestrial environments. The presence of multiple antimicrobial resistant isolates in seawater and oyster samples represent an important public health threat, since seawater is used for recreational purposes and oysters flesh may be consumed raw, and both might be sources of transmission of antibiotic resistant bacteria.

In conclusion, our study shows the occurrence of enteric environmental isolates, displaying multiple antimicrobial resistances in a Caribbean coastal area that could be transmitted to other environments, including human domain. In this context, we present evidences to bring the public attention to this environmental issue.

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