Preventive Veterinary Medicine 218 (2023) 105978

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Detection of *Escherichia coli* and other Enterobacteriales members in seabirds sampled along the Brazilian coast

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ARTICLE INFO

Keywords:
Escherichia coli
Seabirds
Enterobacteriales
Pathotypes
Antimicrobial resistance
EAEC

ABSTRACT

Escherichia coli, an Enterobacterales member, is a normal representative of the microbiota of homeothermic animals. Most strains are commensal, but several pathotypes can cause disease, and numerous antimicrobial resistance factors have been identified. These bacteria have spread rapidly in recent years, highlighting the importance of screening the environment and non-human reservoirs for virulent strains and/or those presenting resistance factors, in addition to other microorganisms of public health importance. In this context, this study aimed to survey Enterobacteriales present in seabirds sampled along the Brazilian coast, comparing findings between migratory and resident birds, as well as between wrecked and non-wrecked animals. *Escherichia coli* pathotypes were also characterized through rapid seroagglutination and polymerase chain reaction techniques and antimicrobial resistance profiles were investigated through the disc agar diffusion method. Cloacal, ocular, oral, tracheal, and skin lesion swabs, as well as fresh feces, were collected from 122 seabirds. The findings indicate these animals as important hosts for opportunistic human pathogens. *Escherichia coli* strains were identified in 70 % of the analyzed seabirds, 62 % of which displaying resistant or intermediate profiles to at least one antimicrobial, while 7% were multiresistant. Resistance to tetracycline (22 %), nalidixic acid (15 %), trimethoprim-sulfamethozaxol (14 %) and ampicillin (12 %) were the most prevalent. Resistance to cefoxitin, a critically important antimicrobial for human medicine, was also detected. Virulence genes for one of the EAEC, ETEC or EPEC pathotypes were detected in 30 % of the identified strains, the first two described in seabirds for the first time. The EAEC gene was detected in 25 % of the sampled seabirds, all resident, 8 % of which exhibited a multidrug-resistant profile. Thus, seabirds comprise important reservoirs for this pathotype. *Escherichia coli* was proven an ubiquitous and well-distributed bacterium, present in all evaluated bird species and sampling sites (except Marajó Island). According to the chi-square test, no significant differences between *E. coli* prevalences or antimicrobial resistance profiles between migratory and resident and between wrecked and non-wrecked

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https://doi.org/10.1016/j.prevetmed.2023.105978
Received 10 January 2023; Received in revised form 13 July 2023; Accepted 19 July 2023

seabirds were observed. Thus, migratory birds do not seem to contribute significantly to *E. coli* frequencies, pathotypes or antimicrobial resistance rates on the Brazilian coast.

1. Introduction

Escherichia coli, an Enterobacterales member, is a representative of the normal intestinal microbiota present in both humans and other homeothermic animals (Murphy et al., 2021). However, certain *E. coli* pathotypes can lead to disease, such as diarrhea (Yang et al., 2017) or extra-intestinal diseases (Jesser and Levy, 2020). Epidemiological studies to identify these pathotypes are, therefore, paramount to better understand diseases caused by this bacterium and improve health service interventions (Jesser and Levy, 2020). Eight diarrheagenic *E. coli* pathotypes have been identified to date, based on their clinical, epidemiological and virulence characteristics, as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), Shiga toxin-producing *E. coli* (STEC), invasive adherent *E. coli* (AIEC) and hemorrhagic enteroaggregative *E. coli* (EAHEC), which carries genes associated with both STEC and EAEC virulence (Farfán-García et al., 2016; Fratamico et al., 2016). These pathotypes cause watery diarrhea, abdominal pain, nausea, vomiting, and fever and, in some cases, diarrhea accompanied by the presence of blood (EHEC and EIEC) or mucus (EIEC) (Yang et al., 2017).

The ETEC, EPEC and EIEC pathotypes are commonly diagnosed in children, mainly in developing countries. ETEC is also responsible for travelers' diarrhea in endemic regions (Yang et al., 2017; Jesser and Levy, 2020). The EPEC pathotype, in particular, can cause persistent infections, requiring the use of antibiotics (Yang et al., 2017), which may become an issue, as resistance to several antimicrobials has been reported for EPEC *E. coli* strains (Langendorf et al., 2015; Malvi et al., 2015).

STEC members are Shiga toxin producers. EHEC is a STEC subtype, commonly associated with foodborne illnesses (Jesser and Levy, 2020) at small infective doses and contact with infected animals (Yang et al., 2017). This subtype can also cause hemolytic uremic syndrome (HUS), a post-infectious complication present in around 10% of affected children (Gilligan, 1999; Yang et al., 2017).

The EIEC pathotype presents a higher infecting dose than the EHEC, with symptoms potentially leading to death and complications, such as megacolon, intestinal perforation, peritonitis, pneumonia and Hemolytic Uremic Syndrome (HUS) (Yang et al., 2017). EAEC is an emerging diarrheal and extra-intestinal pathogen present in both developing and developed countries and affecting all age groups. Asymptomatic carriers are common (Jesser and Levy, 2020).

DAEC comprises a heterogeneous group presenting variable virulence, with AIEC isolated from the intestinal mucosa of individuals suffering from Crohn's disease and chronic diarrhea (Farfán-García et al., 2016). The EAHEC pathotype probably arose from the combination of EHEC and STEC virulence factors, increasing strain virulence potential (Fratamico et al., 2016).

Many of these pathotypes, some displaying antimicrobial resistance markers, are found in wild animals. These bacteria have spread rapidly in recent years, highlighting the importance of environmental and non-human reservoir screening (Murphy et al., 2021). Within a One Health context, antimicrobial resistance comprises one of the greatest human health threats of the 21st century (Ewbank et al., 2021), with wildlife of paramount significance in this context, i.e., regarding the dissemination of pathogenic and multiresistant bacteria, such as migratory birds (Islam et al., 2021).

In this regard, it has been suggested that animals are able to contaminate the environment with *E. coli* pathotypes (McAuley et al., 2014), as well as other Enterobacteriales displaying public health interest (Stenkat et al., 2014). Seabirds, for example, are extremely useful as bacterial pathogen and antimicrobial resistance sentinels (Ewbank et al., 2022). Thus, this study aimed to survey Enterobacteriales present in seabirds along the Brazilian coast, comparing migratory and resident and wrecked and non-wrecked individuals and characterizing the detected *E. coli* strains in terms of virulence and antimicrobial resistance profiles.

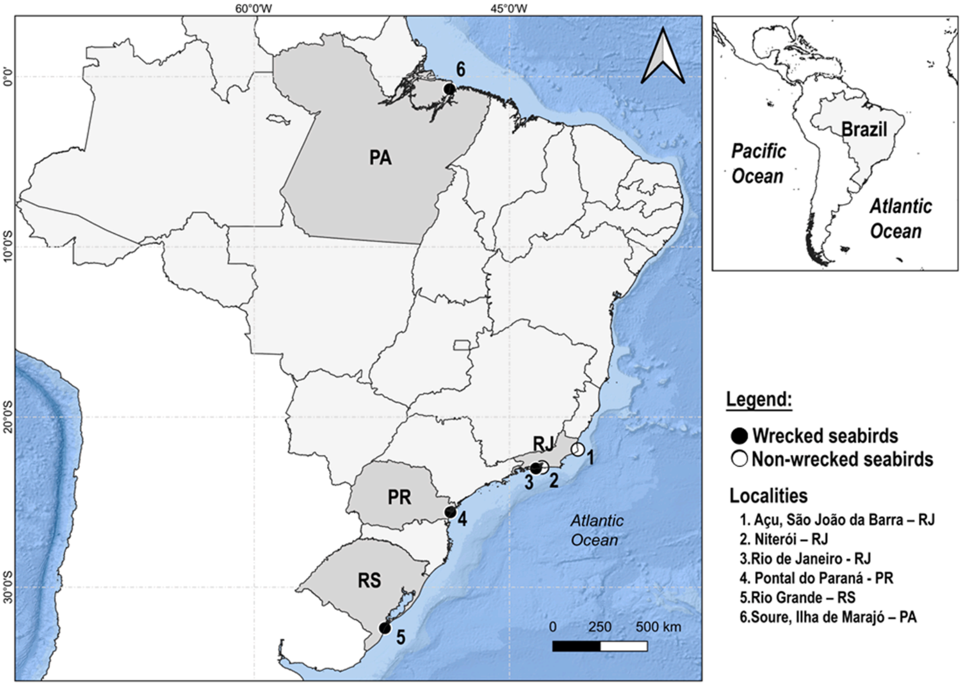

Fig. 1. Sampling point locations of migratory and resident wrecked and non-wrecked seabirds along the Brazil coast.

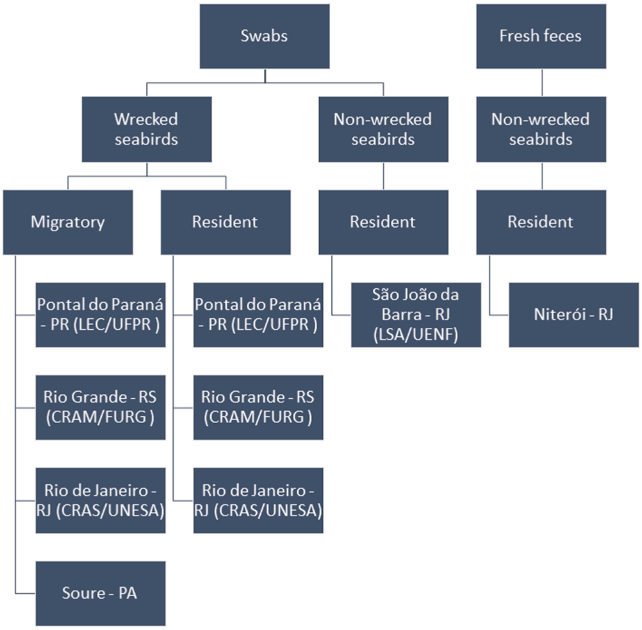

Fig. 2. Sampling methods applied to Enterobacteriales assessment in seabirds throughout Brazilian coast in 2015 and 2016.

2. Material and methods

2.1. Target population

The samples were obtained through opportunistic sampling, i.e., whatever migratory or resident seabirds that the researchers could access in the study area in 2015 and 2016, due to their availability, and not by statistical criteria (Guimarães, 2008). All samplings were approved by the Oswaldo Cruz Institute Ethics Committee on Animal Use (CEUA/IOC: L-005/2016). Individuals found weakened on beaches, unable to swim or fly, were categorized as wrecked, while apparently healthy individuals with no swimming or flying impairment were classified as non-wrecked.

2.2. Study Area

Samples were obtained from seabirds from several Brazilian coast regions, namely Niterói, Rio de Janeiro, Açu (São João da Barra), all three in the state of Rio de Janeiro (RJ), Pontal do Paraná, Paraná (PR), Rio Grande, in the state of Rio Grande do Sul (RS) and Soure, Marajó Island, in the state of Pará (PA) (Fig. 1) in 2015 and 2016. Areas in the state of Rio de Janeiro were selected for being traditionally assessed by our group and for being important stopover sites for migratory birds and greater productivity areas for resident birds foraging. Areas in other states were selected by our working partners.

2.3. Biological sampling

Swabs and fresh feces were collected for this assessment. Swabs were collected from wrecked migratory and resident seabirds and non-wrecked resident seabirds; and fresh feces were collected from non-wrecked resident seabirds. For better understanding of the following sampling strategy, it is depicted in Fig. 2. Some samples were obtained through partnerships with institutions, listed below. There may be differences between sampling methods (cloacal or fecal swabs), however, this difference appears to be more quantitative than qualitative (Stanley et al., 2015), which does not preclude this research, aimed at identifying bacterial species, not counting them.

2.4. Cloacal, skin lesion, and other body part swabs from resident and migratory wrecked seabirds

Samplings were conducted by the Center for Sea Studies Ecology and Conservation Laboratory, at the Federal University of Paraná (LEC/UFPR, PR), the Marine Animal Recovery Center at the University of Rio Grande Foundation (CRAM/FURG, RS), and the Wild Fauna Recovery Center at Estácio de Sá University (CRAS/UNESA, RJ), all following the same sampling protocol.

Opportunistic cloacal swabs, and in some cases, eye, oral, tracheal and/or skin lesion swabs of both migratory and resident seabirds were obtained from individuals found still alive on beaches during the systematic monitoring carried out by the participating institutions or at their arrival, prior to antibiotic therapy, at the participating fauna rehabilitation centers. The swabs were maintained at room temperature in Cary-Blair transport medium until sent to the analytical laboratory. All samples were obtained in duplicate to ensure analytical replication.

B) Cloacal swabs from resident non-wrecked seabirds.

Cloacal swabs from *Sterna hirundinacea* and *Thalasseus acuflavidus* nestlings in Açu, São João da Barra (RJ) were obtained in duplicate following the same collection and transport protocol described in the previous section (SISBIO License – ICMBio n°32295–5), in collaboration with the Laboratory of Animal Health of the Center for Agricultural Sciences and Technologies at the North Fluminense Darcy Ribeiro State University (LSA/UENF).

C) Fresh feces from resident non- wrecked seabirds.

Fresh feces were obtained immediately after excretion in duplicate from resident Guanabara Bay species in the municipality of Niterói (RJ). All were free-living, non-wrecked and apparently healthy birds. The samples were maintained at room temperature in Cary-Blair transport medium until sent to the analytical laboratory.

Table 1

Genes, primer sequences and product sizes employed in the identification of *Escherichia coli* pathotype virulence genes.

Pathotype	Gene	Sequence	Amplicon
Enteroinvasive (EIEC)	*ial*	F 5′-GGTATGATGATGATGAGTGGC-3′ R 5′-GGAGGCCAACAATTATTTCC-3′	630 bp
Enterohemorrhagic (EHEC)/Enteropathogenic (EPEC)	*eaeA*	F 5′-CTGAACGGCGATTACGCGAA-3′ R 5′-GACGATACGATCCAG-3′	917 bp
Enteroaggregative (EAEC)	*eagg*	F 5′-AGACTCTGGCGAAAGACTGTATC-3′ R 5′-ATGGCTGTCTGTAATAGATGAGAAC-3′	194 bp
Enterohemorrhagic (EHEC)	*Stx1*	F 5′-ACACTGGATGATCTCAGTGG-3′ R 5′-CTGAATCCCCCTCCATTATG-3′	614 bp
Enterohemorrhagic (EHEC)	*Stx2*	F 5′-CCATGACAACGGACAGCAGTT-3′ R 5′-CCTGTCAACTGAGCACTTTG-3′	779 bp
Enterotoxigenic (ETEC)	*LT*	F 5′-GGCGACAGATTATACCGTGC-3′ R 5′-CGGTCTCTATATTCCCTGTT-3′	450 bp
Enterotoxigenic (ETEC)	*ST*	F 5′-TTTCCCCTCTTTTAGTCAGTCAACTG-3′ R 5′-GGCAGGATTACAACAAAGTTCACA-3′	160 bp

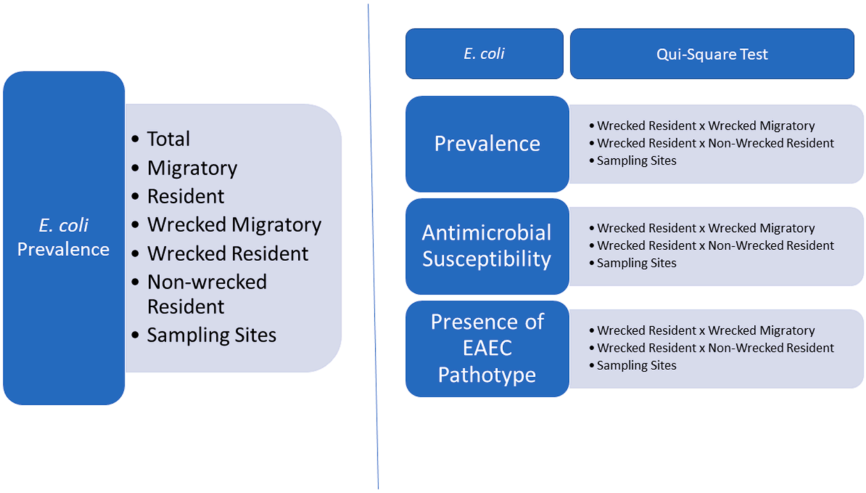

Fig. 3. Statistical analyzes applied to *E. coli* assessment in seabirds throughout Brazilian coast in 2015 and 2016.

2.5. Laboratory analysis

The feces samples and swabs were sent to the National Bacterial Enteroinfections Reference Laboratory at the Oswaldo Cruz Institute, (LRNEB/IOC/FIOCRUZ) in Cary-Blair transport medium, where culturing, strain isolation, biochemical identification, antimicrobial susceptibility testing and antigenic *E. coli* characterization, as well as the isolation and biochemical identification of other Enterobacteriales, were conducted. For logistical reasons, the culture, isolation and biochemical identification of the samples of 58 birds were carried out at the LSA/UENF, following the same methodology applied at the LRNEB.

The samples were first inoculated in enrichment media consisting of Tetrathionate Broth (Merck) and Rappaport-Vassiliadis (Oxoid) and incubated at 37ºC ± 2 and 42ºC ± 2 respectively, for 18–24 h. Isolation was then performed in Eosin methylene blue (EMB) medium (Oxoid), with incubation carried out at 37 °C for 18–24 h. Suspected colonies were transferred to the Costa & Vernin (CV) screening medium (Oxoid), followed by biochemical tests for enterobacteria isolation and identification according to Costa and Hofer (1972).

Antigenic profiling was determined for all strains through the rapid seroagglutination technique on slides containing poly and monovalent somatic antisera for EPEC, EIEC and EHEC O157 identification according to Ewing (1986). For molecular identification, the DNA of each strain was extracted according Moyo et al. (2007). The strains were subjected to multiplex PCR for evaluation of the presence of genes *mdh* (commensal *E. coli*), *lt* and *st* (ETEC), *ial* (EIEC), *stx1* and *stx2* (STEC), *eagg* (EAEC) and *eaeA* (EPEC) according Omar and Barnard (2014). The genes, primer sequences and product sizes employed in this analysis are displayed in Table 1.

Although we recognize that Avian Pathogenic *E. coli* (APEC) is a relevant disease agent for birds (Saviolli et al., 2016), the focus of our laboratory is on human health. In this assessment, strain monitoring in seabirds aimed to detect virulence mechanisms related to infection in humans, so APEC was not a target pathotype.

Strains presenting positive pathogenicity genes in the polymerase chain reaction (PCR) and all strains from samples collected on the same day were selected for antimicrobial susceptibility profile characterizations by the agar disc diffusion methodology reported in CLSI (2017), investigated the following antibiotics: Ampicillin (AMP), Chloramphenicol (CHL), Tetracycline (TCY), Cefoxitin (FOX), Ceftazidime (CAZ), Streptomycin (STR), Ciprofloxacin (CIP), Gentamicin (GEN), Imipenem (IMP), Nalidixic Acid (NAL), Trimethoprim-Sulfame thoxazole (SXT) and Nitrofurantoin (NIT).

2.6. Statistical analyses

Statistical analyses were performed using the IBM SPSS Statistics Trial Version 26.0 software. The prevalence of each bacterial species

Table 2
General description and number of seabirds investigated concerning the presence of *E. coli* and other Enterobacteriales from different Brazilian coast regions.

Species	Common name	Migratory status	Conservation status	Wrecked		Total	*E. coli* prevalence (%)
				Yes	No		
Ardea alba	Great egret	R	LC	0	8	8	75
Ardea cocoi	Cocoi heron	R	LC	1	0	1	100
Croicocephalus maculipennis	Brown-hooded gull	R	NE	9	0	9	89
Fregata magnificens	Magnificent frigatebird	R	LC	1	0	1	100
Larus dominicanus	Kelp gull	R	LC	3	0	3	100
Leucophaeus atricilla	Laughing gull	VN	LC	1	0	1	0
Macronectes giganteus	Southern giant petrel	VS	LC	1	0	1	0
Nannopterum brasilianus	Neotropic cormorant	R	LC	10	18	28	71
Procellaria aequinoctialis	White-chinned petrel	VS	VU	3	0	3	67
Pterodroma incerta	Atlantic petrel	VS	EN	1	0	1	0
Spheniscus magellanicus	Magellanic penguin	VS	LC	14	0	14	71
Sterna hirundinacea	South American tern	R	LC	0	12	12	92
Sula leucogaster	Brown booby	R	LC	26	0	26	58
Thalassarche melanophris	black-browed albatross	VS	LC	1	0	1	100
Thalasseus acuflavidus	Cabot's tern	R	NE	0	13	13	54
Total	15 species	R= 9; VS= 5; VN= 1	EN= 1; VU= 1; LC= 11; NE= 2	71	51	122	70

R = Resident; VS = Seasonal Visitor from the Southern Hemisphere; VN = Seasonal Visitor from the Northern Hemisphere (Piacentini et al., 2015). EN = Endangered; VU = Vulnerable; NT = Near Threatened; LC = Least Concern; NE = Not Evaluated (IUCN, 2022).

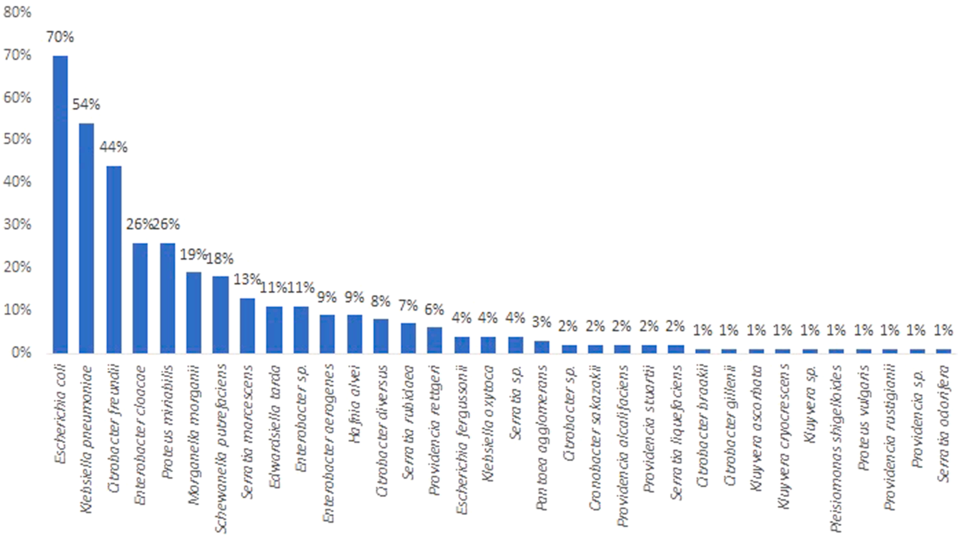

Fig. 4. Prevalence of identified Enterobacteriales in migratory and resident wrecked and non-wrecked seabirds, sampled off the Brazilian coast in 2015 and 2016.

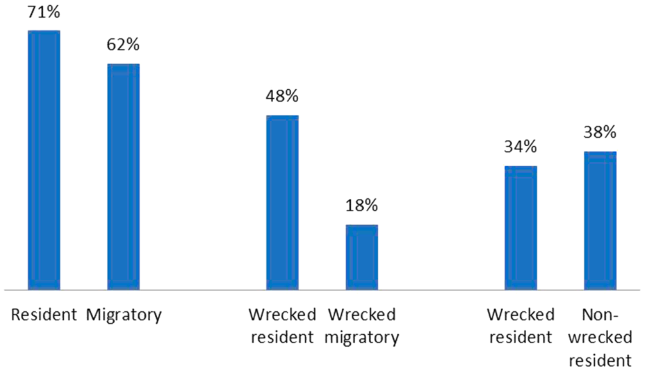

Fig. 5. Prevalence of *E. coli* in seabirds sampled off the Brazilian coast in 2015 and 2016, according to migratory status, migratory status of wrecked birds, and resident wrecked or non-wrecked status.

concerning the total study population was first calculated. Then, only *E. coli* data were treated, calculating the prevalence for each seabird group (total migratory and residents, wrecked migratory and residents and wrecked and non-wrecked residents) and in sampling sites. Antimicrobial susceptibility prevalence rates were also calculated. Comparisons of prevalence rates between resident wrecked seabirds and migratory wrecked seabirds and between resident wrecked seabirds and resident non-wrecked seabirds, and by sampling sites were conducted, followed by comparisons between samplings sites employing Pearson's Chi-Square Test. A p-value ≤ 0.05 was set as significant for all analysis. The Pearson Chi-Square Test was also used to verify potential associations between antimicrobial susceptibility and bird status (resident or migratory) and between antimicrobial susceptibility and wrecked status, as well as between the presence of the EAEC pathotype and resident or migratory status, wrecked status, and in sampling sites. For better understanding, statistical analyzes are depicted in Fig. 3.

3. Results and discussion

Samples were obtained from 122 birds, 51 resident non-wrecked individuals and 71 wrecked animals. Among the wrecked seabirds, 50 were residents and 21 were migratory visitors, all from the southern hemisphere except one, from the northern hemisphere. The evaluated seabird species are listed in Table 2 alongside their *E. coli* prevalence rates. *Ardea alba* (great egret), *A. cocoi* (cocoi heron) and *Nannopterum brasilianus* (Neotropic cormorant), despite not being considered strictly seabirds, were included because they also inhabit and feed in marine environments (Votier and Sherley, 2017), and because their samples were obtained from the coastal zone.

The general prevalence of *E. coli* in the present study was of 70%. The prevalence rates of other Enterobacteriales species are represented in Fig. 4. Fig. 5 displays *E. coli* prevalence rates per group (residents and migratory seabirds, wrecked residents and migratory seabirds, non-wrecked and wrecked resident birds).

Even though no strain was serologically identified herein, these do not necessarily correspond to "non-typable" strains, as they simply cannot be evaluated among the serotypes/serogroups included in the tested antisera panel. Currently, serotyping has given way to molecular techniques for the diagnosis of diarrheagenic *E. coli* infections, allowing for a more discriminatory diagnosis (Costa et al., 2014; Omar and Barnard, 2014).

E. coli was proven ubiquitous and well-distributed among all investigated seabirds. Only three species (*Leucophaeus articilla*, *Macronectes giganteus* and *Pterodroma incerta*) were negative for this bacterium. However, only one specimen of each was assessed.

E. coli is part of the normal microbiota of seabirds and prevalence rates compatible with those calculated herein have been reported in São Paulo, Brazil (Saviolli et al., 2016), in the USA (Steele et al., 2005; Atterby et al., 2016) and in Morocco (Barguigua et al., 2019), while lower prevalence rates have been reported in seabirds in Santa Catarina, Brazil (Ebert et al., 2016), in Bangladesh (Hasan et al., 2014), in coastal birds and seabirds in Europe (Stenkat et al., 2014; Giacopello et al., 2016; Zendri et al., 2020), in Mexico (Contreras-Rodríguez et al., 2019), in Australia (Lundbäck et al., 2021) and in Peru (Espinoza et al., 2021). Higher rates have been reported in gulls in the Czech Republic (Dolejská et al., 2009) and Italy (Russo et al., 2021).

The most prevalent Enterobacterales species identified herein were *Klebsiella pneumoniae* (54 %), *Citrobacter freundii* (44 %), *Enterobacter cloacae* (26 %), *Proteus miriabilis* (26 %), *Morganella morganii* (19 %), *Serratia marcescens* (13 %); *Edwardsiella tarda* (11 %). Furthermore, *Pseudomonas aeruginosa* was also detected in 39 % of the isolates.

E. cloacae, *P. aeruginosa* and *K. pneumoniae* have also been reported as among the most prevalent species in waterfowl in Germany by Stenkat et al. (2014), who correlated the presence of enterobacteria in these birds with piscivorous diets. In this regard, piscivorous birds are excellent bacteria sentinels, as they may feed on the same fish species as humans.

The most prevalent bacteria identified in the present study were also

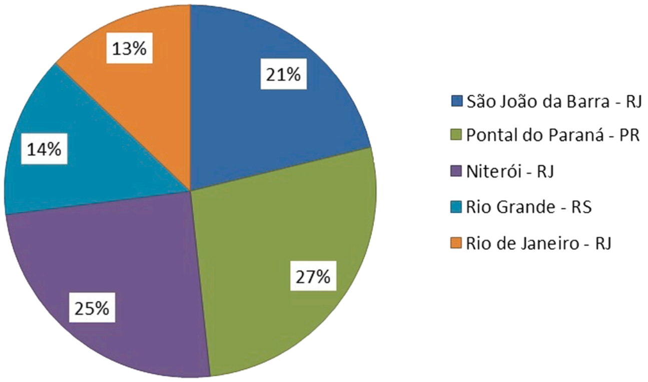

Fig. 6. *E. coli* prevalence rates in seabirds sampled from different Brazilian coast regions in 2015 and 2016.

Table 3
Percentage of susceptible, resistant, and intermediately resistant *E. coli* strains per each tested antimicrobials from seabirds sampled from different Brazilian coast regions in 2015 and 2016.

Antimicrobial	Resistant *E. coli* strains (%)	Intermediary *E. coli* strains (%)
NAL	15	1
CIP	7	0
AMP	12	16
FOX	1	0
CAZ	0	0
IMP	0	0
STR	7	18
GEN	4	0
CHL	0	0
STR	14	0
TCY	22	0
NIT	0	1
Susceptible to all antimicrobials	38	-

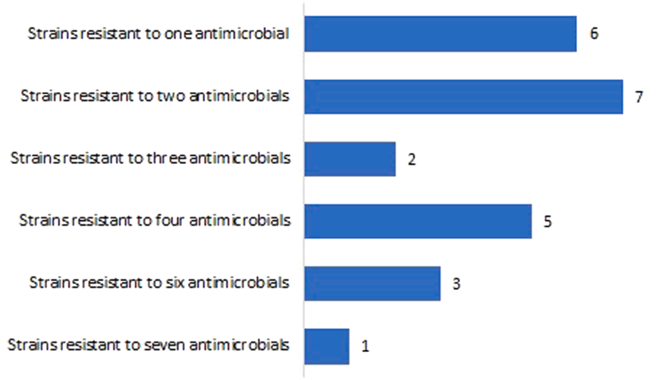

Fig. 7. Number of antimicrobials to which strains isolated from seabirds from the Brazilian coast presented resistance to.

identified in Kelp gulls in Santa Catarina (Brazil) (Ebert et al., 2016) and in seabirds and waterfowl in rehabilitation centers in the USA (Steele et al., 2005) and in Italy (Giacopello et al., 2016), respectively. This suggests that are common species in these animals.

In view of these findings, the evaluated seabirds sampled in Brazil can be considered as opportunistic human pathogen hosts (Stenkat et al., 2014). The most prevalent Enterobacteriales identified herein make up the normal microbiota of humans and, in some situations, can cause respiratory, urinary, wound infections, bacteremia and even septicemia, and are also closely linked to nosocomial infections (Brooks and Carroll, 2010). They are also pathogenic for birds (Stenkat et al., 2014), with *E. tarda* highly noteworthy as inhabiting other aquatic animals and comprising a known seabird pathogen (Abbott and Janda, 2006).

Considering all analyzed birds, no significant associations ($p = 0.620$) between the presence of *E. coli* and migratory status was observed, even when excluding non- wrecked birds, in order to compare status (residents x migratory) only among wrecked birds ($p = 0.395$) and excluding migratory birds in order to compare wrecked versus non-wrecked specimens ($p = 0.470$). This is due to the fact that *E. coli* is a very well-disseminated bacterial species (Atterby et al., 2016). Thus, it seems that migratory birds do not contribute to greater *E. coli* loads to the Brazilian coast, even though some studies point to the importance of migratory seabirds in the dissemination of both pathogenic and multi-resistant antimicrobial *E. coli* (Islam et al., 2021).

E. coli was also ubiquitous in all sampling sites, not detected only at Marajó Island (Fig. 6). We don't believe that the absence of isolation from this location is due to distance from the laboratory, since samples were transported in Cary-Blair media, that was described as the best conservation media compared to others (Dan et al., 1989). Data from this location were, therefore, removed for the chi-square calculation, which indicated no association between *E. coli* prevalence rates and sampling site ($p = 0.414$), further suggesting that this bacterium is well-disseminated throughout the Brazilian coast in all study regions. Similar findings have been reported for seagulls in Alaska (Atterby et al., 2016), with similar *E. coli* prevalence rates detected in both remote and urbanized areas.

3.1. *Antimicrobial susceptibility profile characterization*

The percentages of strains susceptible, resistant, and intermediately resistant to each tested antimicrobial are displayed in Table 3. The number of antimicrobials to which the isolated strains displayed resistance to is depicted in Fig. 7.

The frequency of antimicrobial resistance in *E. coli* detected herein was the same as that reported by Saviolli et al. (2016) for strains isolated from frigates in São Paulo, Brazil, and similar to that reported for gulls from an urbanized area in Alaska (USA) by Atterby et al. (2016), but higher than those found by Dolejská et al. (2009), Bonnedahl et al., (2009, 2010), Alroy and Ellis (2011), Hasan et al. (2014), Stedt et al. (2014), Zendri et al. (2020) and Mukerji et al. (2020) in seabirds from the US, Bangladesh, Europe and Australia. A total of 7% of the isolated strains were multidrug-resistant, a frequency similar to the studies carried out by Saviolli et al. (2016) and Hasan et al. (2014), and lower than those reported by Atterby et al. (2016), Barguigua et al. (2019) and Espinoza et al. (2021).

Resistance to tetracycline, ampicillin, trimethoprim-sulfamethoxazole and nalidixic acid have also been detected in *E. coli* strains isolated from seabirds and shore birds in other studies in Brazil (Saviolli et al., 2016; Ewbank et al., 2021), in other locations in South America (Báez et al., 2015; Espinoza et al., 2021), North America (Alroy and Ellis, 2011; Atterby et al., 2016), Asia (Hasan et al., 2014), Europe (Bonnedahl et al., 2009; Dolejská et al., 2009; Bonnedahl et al., 2010; Radhouani et al., 2009; Stedt et al., 2014; Zendri et al., 2020; Russo et al., 2021), Africa (Barguigua et al., 2019) Oceania (Mukerji et al., 2020) and even the Arctic (Sjölund et al., 2008). This indicates that resistant strains are detected even in remote areas (Sjölund et al., 2008; Ewbank et al., 2021), probably arriving carried by migratory birds (Sjölund et al., 2008). This further demonstrates the importance of investigating the antimicrobial susceptibility of strains isolated from seabirds, as the resistance patterns found herein seem to be disseminated worldwide.

In Brazil, the use of tetracyclines, beta-lactams, sulfonamides, and quinolones as feed additives for production animals is banned (Brasil, 2009), but still occurs (Regitano and Leal, 2010). However, these drugs are still used in veterinary therapeutics and are commonly employed in human medicine (Dougnac et al., 2015; ANVISA, 2021). Furthermore, some, such as tetracyclines, are environmentally persistent, which may explain the development of bacterial resistance (Retamal et al., 2015). The use of these drugs in aquaculture is also important concerning their input into surface waters that, ultimately, reach the oceans (Regitano and Leal, 2010).

Although the low prevalence of cefoxitin resistance, a critically important antimicrobial for human medicine (WHO, 2018) noted worldwide, and its recently described resistance in *E. coli* isolated from seabirds (Mukerji et al., 2020; Zendri et al., 2020; Ewbank et al., 2021; Ewbank et al., 2022). Herein, a strain isolated from a resident juvenile red-billed tern nestling, in São João da Barra, is noteworthy, as the bird has already been contaminated with this strain even before leaving its nest.

Concerning only the pathogenic *E. coli* strains, most (59 %) were resistant to at least one antimicrobial, important concerning the treatment of environmentally contaminated humans. Among the multidrug-resistant strains, 50 % were isolated from cormorants, 17% from Brown-hooded gulls, 17 % from kelp gulls and 17 % from frigates. Free-ranging wild animals are rarely treated with antibiotics (Hasan et al., 2014). Thus, the high prevalence of antimicrobial resistance noted in seabirds suggests an anthropogenic contamination source, due to the high use of antibiotics in humans (Duartea et al., 2002; Ewbank et al., 2021), proven by Atterby et al. (2016) when comparing gulls from remote and urbanized areas in Alaska.

The high prevalence of antimicrobial resistance detected herein may be also associated to inefficient effluent treatment in Brazil. The release of these compounds in ocean environments, considered antimicrobial resistance transmission hotspots, facilitates the selection of resistant strains and the transmission of horizontal bacteria resistance, which, in turn, colonize wild animals (Piotrowska and Popowska, 2015; Masarikova et al., 2016).

All birds presenting multiresistant strains were residents and most were sampled in the state of Rio de Janeiro, where less than half of the municipalities have a sewage collection network (49.2 %) and 41.3 % do not have any sewage treatment at all. A multidrug-resistant strain was also detected in the state of Rio Grande do Sul, where only 15.1 % of municipalities have sewage treatment (IBGE, 2010). These data may, in fact, be overestimated, as they refer only to legal households, while irregular households, in slums, for example, release untreated sewage directly into the sea or into rivers that flow into the sea (Fistarol et al., 2015).

Some seabird species, such as kelp gulls and brown-hooded gulls, present opportunistic habits, feeding on human waste found in garbage and sewage (Ebert and Branco, 2009). They may, therefore, become contaminated and carry multiresistant bacteria (Migura-Garcia et al., 2017). It is noteworthy that a strain isolated from a kelp gull, in Rio Grande, was resistant to seven different antimicrobials (AMP; TCY; STR; CIP; GEN; NAL; SXT). Even birds with no opportunistic habits are in constant contact with multiresistant bacteria-contaminated water, such as cormorants and frigates that inhabit Guanabara Bay, in the state of Rio de Janeiro. This ecosystem receives effluents from 16 municipalities, with an estimated discharge of 18 m^3 /s of untreated sewage. Several resistant and multiresistant bacteria identified in this study have been detected in the Bay, which could be mitigated with adequate effluent treatment (Fistarol et al., 2015). *E. coli* strains resistant to six antimicrobials were isolated from two cormorants from the rehabilitation center in Rio de Janeiro, presenting the same resistance pattern (AMP, TCY, CIP, GEN, NAL, SXT), as well as a frigate, with a very similar pattern (AMP, TCY, STR, CIP, NAL, SXT). The number of strains resistant to ciprofloxacin and nalidixic acid among those isolated from this rehabilitation center in Rio de Janeiro in the same month (83 % of the strains presenting some type of resistance) are also noteworthy, another evidence of increased resistance to fluoroquinolones, noted since the 1980s (Ministério da Saúde, 2011). Ewbank et al. (2022) found an *E. coli* strain resistant to eight antimicrobials in *Fregata magnificens* also in southeastern Brazil, but in an area uninhabited by humans, but displaying a different pattern from the multiresistant strains identified in the present study.

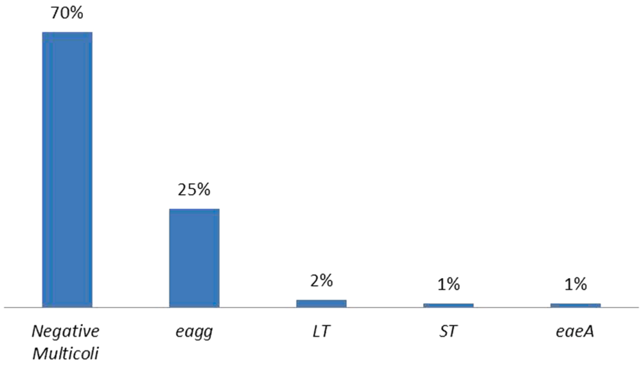

Fig. 8. Identification frequency of *E. coli* virulence genes isolated from seabirds sampled off the Brazilian coast in 2015 and 2016.

The chi-square test indicated no significant association between antimicrobial susceptibility and seabird wrecking, migratory or resident status or sampling site, once again suggesting a wide distribution of antimicrobial resistance in all groups.

3.2. Molecular characterization

A total of 70 % of the isolated *E. coli* strains were negative for the investigated pathogenicity genes, while 25 % were positive for the *eagg* gene, characterizing the EAEC pathotype, 2 % were positive for *LT* (ETEC); and 1% for *ST* (ETEC) and *eaeA* (EPEC) (Omar and Barnard, 2014) (Fig. 8). *E. coli* is ubiquitous and frequently found in seabirds, but the presence of pathogenicity genes characterizes the seabirds investigated herein pathogenic *E. coli* reservoirs. Of the three identified pathotypes, only EPEC has been previously reported in seabirds, in the USA (Steele et al., 2005) and in Japan (Kobayashi et al., 2009). Thus, to the best of our knowledge, the present study is the first to report ETEC and EAEC in seabirds.

With 25 % positivity for the *eagg* gene, we can consider that seabirds are important reservoirs of the EAEC pathotype. Despite never having been diagnosed in seabirds before, the high prevalence of this pathotype can be explained by the fact that it is the most widespread, present both in developing and developed countries and in all age groups (Jesser and Levy, 2020). In some parts of the world, EAEC is the main bacterial pathogen associated to diarrhea, with food comprising the main contamination route. It is also more commonly linked to antimicrobial resistance than other pathotypes (Okhuysen and Dupont, 2010).

Most of the EAEC strains identified herein were intermediately resistant (42 %) or sensitive (38 %) to the tested antimicrobials. However, it is noteworthy that 8 % of the isolates were multidrug-resistant, including the previously discussed strain resistant to six antimicrobials from the cormorant sampled in Rio de Janeiro and the one isolated from a brown-hooded seagull sampled at the Rio Grande (RS) landfill. Once again, the importance of studying species with opportunistic habits that feed on human waste is highlighted (Migura-Garcia et al., 2017).

A juvenile non-wrecked south American tern from the nest of Açu, São João da Barra (RJ) was contaminated by the ETEC pathotype even before leaving its nest. The ETEC pathotype was also identified in a brown booby wrecked in the metropolitan region of Rio de Janeiro. The bird contaminated by an EPEC strain was a frigate, also wrecked in the metropolitan region of Rio de Janeiro.

The EPEC and ETEC pathotypes verified herein are important pathotypes identified in human samples from developing countries, such as Brazil, comprising mainly diarrhea agents in children (Jesser and Levy, 2020). ETEC is significant in the study region, as it is the main pathogen involved in cases of travelers' and children's diarrhea in Latin America, acquired through the intake of contaminated water and food (Yang

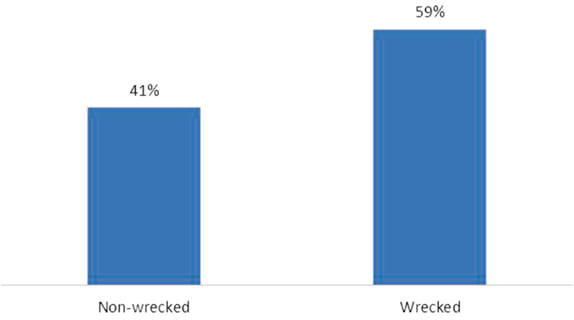

Fig. 9. Frequency of identification of the *eagg* gene isolated from non-wrecked and wrecked resident birds sampled off the Brazilian coast in 2015 and 2016.

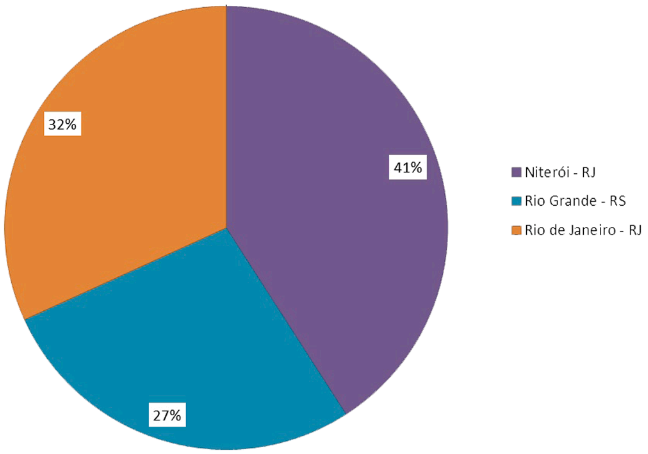

Fig. 10. Distribution of the *eagg* gene in *E. coli* strains isolated from seabirds off the Brazilian coast in 2015 and 2016.

et al., 2017). EPEC is the pathotype most commonly associated to foodborne illness outbreaks, followed by ETEC and EAEC (Yang et al., 2017). The first two been previously reported in fish (Harada et al., 2013; Park et al., 2014), indicating the importance of monitoring aquatic environments, with seabirds as sentinels. It is noteworthy that all the described genes were only detected in resident birds. The case of the juvenile infected with a pathogenic strain even before leaving the nest draws attention, as these animals can be contaminated at such a young age with pathogenic strains through interaction with their parents (feces or regurgitated food) or even with the soil (Migura-Garcia et al., 2017) or vertical transmission via egg (Teixeira et al., 2013).

The *eagg* gene displayed the highest frequency and was only identified in resident birds. Of these, 59 % were wrecked and 41 % were non-wrecked (Fig. 9).

The Pearson's chi-square test was performed, as the *eagg* gene displayed a high prevalence in the study population. As the gene was only identified in resident birds, a significant association ($p = 0.033$) was found between status (migratory x resident) and gene identification, as expected. No association was identified regarding wreck status, ($p = 0.762$), since the results are well balanced (Fig. 9).

The *eagg* gene was only identified in Niterói (RJ), Rio Grande (RS) and Rio de Janeiro (RJ) (Fig. 10). Considering only these locations, the Pearson's chi-square test indicated no association between sampling location and the *eagg* gene ($p = 0.811$), suggesting that the EAEC pathotype is well disseminated in these locations.

4. Conclusions

Seabirds were proven to be important hosts of opportunistic human pathogens, especially *E. coli* (70 %), *Klebsiella pneumoniae* (54 %), *Citrobacter freundii* (44 %), *Pseudomonas aeruginosa* (39 %), *Enterobacter cloacae* (26 %), *Proteus miriabilis* (26 %), *Morganella morganii* (19 %), *Serratia marcescens* (13 %) and *Edwardsiella tarda* (11 %).

A total of 62 % of the isolated *E. coli* strains were resistant or intermediately resistant to the tested antimicrobials and 7 % were multidrug-resistant. Among these, one strain was resistant to seven antimicrobials and three strains to six. Resistance to tetracycline (22 %), nalidixic acid (15%), trimethoprim-sulfamethozaxol (14 %) and ampicillin (12 %) were the most prevalent. Although resistance to cefoxitin was low (1 %), this is a critically important antimicrobial for human medicine and has only recently been identified in strains isolated from seabirds.

This study is the first to describe ETEC and EAEC in seabirds, the latter positive in 25 % of the sampled birds, all resident (8 % of which were multiresistant), suggesting their importance as a reservoir for these pathotype. EPEC was also detected.

E. coli was proven a ubiquitous and well-distributed bacterium in all investigated seabird species, as well as in all study sites (except Marajó Island), with no differences in prevalence rates or resistance to antimicrobials detected between migratory and resident birds and between wrecked and non-wrecked migratory and non-migratory birds. Thus, migratory birds do not seem to significantly contribute to higher *E. coli* loads along the Brazilian coast. Both groups contribute equally to pathotypes and strains with important bacterial resistance in human medicine.

Funding

This study was partially financed by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001.

Declaration of Competing Interest

We have no conflicts of interest to disclose.

Acknowledgements

The authors would like to thank the National Reference Laboratory for Bacterial Enteroinfections (LRNEB/IOC/FIOCRUZ), employees and scholarship holders, including those responsible for the culture media manufacture, and the CCTA Animal Health Laboratory bacteriology sector at UENF. Thanks are also due to the CCTA Animal Health Laboratory virology sector at UENF, the Ecology and Conservation Laboratory - Mammals and Sea Turtles at the UFPR Center for Marine Studies, the FURG Marine Animal Recovery Center, and the Estácio de Sá University Wild Fauna Recovery Center for their sampling assistance. We also thank Greicy F. Ruenes who kindly prepared the map used in this study.

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