Research Paper

Isolation of *Escherichia coli* and *Salmonella* spp. from free-ranging wild animals

Renata de Oliveira Iovine[1], Catia Dejuste[2,3], Flávia Miranda[2], Claudia Filoni[1,3,4],
Marina Galvão Bueno[3,5], Vania Maria de Carvalho[1]

[1]Laboratório de Biologia Molecular e Celular, Universidade Paulista, São Paulo, SP, Brazil.
[2]Projeto Tamanduá, São José dos Pinhais, PR, Brazil.
[3]Instituto Brasileiro para Medicina da Conservação, Recife, PE, Brazil.
[4]Laboratório de Diagnóstico Molecular, Universidade Estadual Paulista "Julio de Mesquista Filho",
Botucatu, SP, Brazil.
[5]Laboratório de Patologia Comparada de Animais Selvagens, Universidade de São Paulo, São Paulo,
SP, Brazil.

Abstract

Increasing interactions between humans, domestic animals and wildlife may result in inter-species transmission of infectious agents. To evaluate the presence of pathogenic *E. coli* and *Salmonella* spp. and to test the antimicrobial susceptibility of isolates, rectal swabs from 36 different free-ranging wild mammals were taken from two distinct natural sites in Brazil: Cantareira State Park (CSP, state of São Paulo) and Santa Isabel do Rio Negro Region (SIRNR, state of Amazonas). The swabs were randomly collected and processed for bacterial isolation, identification, characterization and antimicrobial resistance. Eighteen *E. coli* strains from CSP and 20 from SIRNR were recovered from 14 and 22 individuals, respectively. Strains from animals captured in CSP, the site with the greatest anthropization, exhibited a higher range and percentage of virulence genes, including an *eae+/bfpA+* strain. Antimicrobial resistance was verified in strains originating from both sites; however, in strains from SIRNR, aminopenicillins were almost the exclusive antimicrobial class to which strains exhibited resistance, whereas in CSP there were strains resistant to cephalosporins, sulfonamide, aminoglycoside, tetracycline and fluoroquinolone, in addition to strains exhibiting multidrug resistance. Two strains of *Salmonella enterica* that are known to be associated with reptiles, serotypes Belem and 60:r:e,n,z15, were recovered only from Amazonian animals and showed susceptibility to all classes of antimicrobials that were tested. Although the potential impact of these pathogens on wildlife remains unknown, bacteria isolated from free-ranging wild animals may provide relevant information about environmental health and should therefore be more deeply studied.

Key words: *E. coli*, *Salmonella*, wild mammals, One Health, Brazil.

Introduction

Wildlife plays an important role within the context of the One World, One Health concept. Wild animals can serve as sentinels for infectious diseases and illnesses resulting from environmental contamination with toxic substances; however, wildlife can also transmit pathogens to other animal species (directly and indirectly through environmental dispersal), including humans (Rabinowitz *et al.*, 2009). *Escherichia coli* is one of the primary intestinal commensal organisms found in endothermic animals and therefore it is widely disseminated in the environment. Investigating potential pathogenic *E. coli* strains and/or antibiotic resistance may provide information regarding human activity in select ecological niches. Studies have indicated that living in proximity to humans may alter the *E. coli* populations of wildlife intestinal microbiota (Rwego *et al.*, 2008; Gordon and Cowling, 2003), which can in turn serve as a reservoir of *E. coli* strains that are pathogenic to humans (Bélanger *et al.*, 2011).

Send correspondence to: V.M. Carvalho. Av. José Maria Whitaker 290, 04057-000 São Paulo, SP, Brazil. E-mail: vaniamc@uol.com.br.

Bacteria belonging to the *Salmonella* genus are recognized as important primary zoonotic pathogens. Unlike *E. coli*, *Salmonella* spp. are capable of persisting in aquatic environments for long periods of time, even in locations with high levels of eutrophication. The ubiquitous nature of *Salmonella* permits a cyclic exchange between host and environment (Winfield and Groisman, 2003). Thus, wildlife can serve as reservoirs of diverse serotypes, especially with respect to reptilian species (Winfield and Groisman, 2003; Hoelzer *et al.*, 2011).

Antimicrobial resistance in bacteria isolated from individuals with no history of previous exposure to antimicrobial agents is indicative of the environmental dissemination of resistant strains and residual antimicrobial products through effluents from urban areas and animal production units (Costa *et al.*, 2013). The present study was conducted to evaluate the presence of pathogenic *E. coli* and *Salmonella* spp. and to test antimicrobial susceptibility in isolates taken from fecal samples of free-ranging wild mammals in two distinct natural sites in Brazil.

Materials and Methods

Animals and biomes

A total of 14 different wild mammals (Table 1) from Cantareira State Park (CSP) (10 coati, 3 opossum, and 1 grey short-tailed opossum) and 22 (Table 2) from the Santa Isabel do Rio Negro Region (SIRNR) (18 bats, 3 rodents and 1 marsupial), all of them apparently healthy, were randomly captured using traps and were chemically restrained in strict accordance with protocols approved by the Research Ethics Committee (Permit Number: 140/12 CEP/ICS/UNIP). All activities were performed in full compliance with federal permits issued by the Brazilian Ministry of Environment (Permit Numbers: CSP-260108-000.327/0 2008; SIRNR- SISBIO 27923-4). CSP (23°26'58.84" S; 46°38'7.94" O) is located in one of the world's largest native areas of Atlantic forest within a metropolitan area (the city of São Paulo, which is the largest metropolis in South America), allowing for the possibility of close interactions between local fauna and visitors and domestic animals inhabiting the surrounding areas. In contrast, SIRNR (0°24'51.61" S; 65° 1'10.23" O) is located in Amazonia and is accessible only via fluvial transportation. Human population density is low in this region.

Bacterial isolation and identification

Two rectal swabs were taken per animal. The swabs were delicately introduced into the rectum through rotational movements after cleaning the perianal area with gauze and sterile saline. The swabs were then immediately placed in Stuart transport medium (COPAN Venturi Transystem®) and kept under refrigeration. The first swab that was collected was seeded onto MacConkey Agar (Difco) to isolate *E. coli*, and the resulting colonies were identified using Enterokit B (Probac do Brasil). To isolate *Salmonella* spp., the second fecal swab sample was processed according to a previously published protocol (Michael *et al.*, 2003). Colonies suggestive of *Salmonella* spp. were confirmed using an API 20E identification system (BioMerieux), and they were serotyped in the Reference Center for *Salmonella* Serotyping (Instituto Adolfo Lutz, São Paulo, SP, Brazil).

E. coli virulence genotype and phylogenetic analysis

Strains identified as *E. coli* that were phenotypically different from each other were frozen in Brain Heart Infusion Medium (Difco) with 80% glycerol [Invitrogen; 1:1(v/v)] at -80 °C for further analysis. A PCR assay was used to test the isolates for the presence of virulence genes (VGs) known to be associated with diarrheagenic *E. coli* (DEC) strains (*eae, bfpA, stx1* and *stx2*) (Pollard *et al.*, 1990; Olsvik *et al.*, 1991; Gannon *et al.*, 1993; Gunzburg *et al.*, 1995) and with extraintestinal pathogenic *E. coli* (ExPEC) (*papC*, *papEF*, *sfa*, *fyuA*, *iucD*, *hly*, *cnf1*, *cvaC*, *traT* and *malX*) (Le Bouguenec *et al.*, 1992; Johnson and Stell, 2000). Phylogenetic groups were determined using an established PCR-based method for assessing *chuA* and *yjaA* genes and the TSPE4.C2 DNA fragment (Clermont *et al.*, 2000).

An EDL 933 strain was used as a positive *E. coli* control for *eae, stx1*, and *stx2* genes, and an E2348/69 strain was used as the control for the *bfpA* gene. For ExPEC genes, C7, JJ079 and BUTI 1-7-6 strains were used as positive controls in all PCR reactions, and the JJ055 (derived from K12) strain was used as a negative *E. coli* control (all were provided courtesy of Prof. Dr. James R. Johnson, University of Minnesota). The controls used for phylogenetic determination were JJ055 (Group A), EC 15 (Group B1), JJ079 (Group B2), and EDL 933 (Group D).

Antimicrobial susceptibility test

Antimicrobial susceptibility was assessed using a disk diffusion method according to CLSI protocols (CLSI, 2013a; CLSI, 2013b). The following antimicrobials were tested: the amoxicillin (10 µg) and ampicillin (10 µg) aminopenicillins; the cephalexin (30 µg), cefoxitin (30 µg) and ceftiofur (30 µg) cephalosporins; the ciprofloxacin (5 µg) and enrofloxacin (5 µg) fluoroquinolones; the amphenicol chloramphenicol (30 µg); the combined sulfonamide sulfametaxazol + trimethoprim (25 µg); the streptomycin (10 µg) and gentamicin (10 µg) aminoglycosides; and tetracycline (30 µg). Strains resistant to three or more classes of antimicrobials were considered to be multidrug resistant (MDR) (Schwarz *et al.*, 2010).

Table 1 - Virulence genes, phylogenetic grouping and antimicrobial resistance in *E. coli* strains isolated from free-ranging wild mammals from Cantareira State Park.

Animal species	Strain	Virulence genes														Phylogenetic group				Antimicrobial resistance[1]
		eae	bfp	stx1	stx2	papC	papEF	sfa	fyuA	iucD	hly	cnf1	cvaC	traT	malX	A	B1	B2	D	
coati (*Nasua nasua*)	1/1						x		x		x		x	x					x	Susceptible
	1/2												x	x	x				x	Amp
coati (*Nasua nasua*)	2/1					x											x			Amp
	2/2	x														x				Amp, Amo
coati (*Nasua nasua*)	3	x								x		x		x		x				Amp, Amo, Str, Tet, Sxt, Cip[2]
coati (*Nasua nasua*)	4	x											x	x			x			Amp
coati (*Nasua nasua*)	5	x												x				x		Susceptible
coati (*Nasua nasua*)	6/1					x		x	x		x		x	x				x		Amp
	6/2					x			x					x	x				x	Amp, Amo, Cfx, Tet, Sxt[2]
coati (*Nasua nasua*)	7/1													x			x			Amp
	7/2								x						x			x		Amp
coati (*Nasua nasua*)	8								x					x			x			Amp, Cfx
coati (*Nasua nasua*)	9/1													x					x	Amp, Amo, Cfe, Str, Sxt[2]
	9/2	x	x											x				x		Susceptible
opossum (*Didelphis marsupialis*)	10								x					x	x			x		Amp, Sxt
opossum (*Didelphis marsupialis*)	11								x					x		x				Amp, Tet, Sxt
opossum (*Didelphis marsupialis*)	12								x					x		x		x		Amp, Amo
grey short-tailed opossum (*Monodelphis domestica*)	13	x					x									x				Amp, Amo

[1]AMP: ampicillin, AMO: amoxicillin, CIP: ciprofloxacin, STR: streptomycin, CFE: cephalexin, CFX: cefoxitin, TET: tetracycline, SXT: trimethoptim-sulfamethoxazole.
[2]Multidrug resistant.

Table 2 - Virulence genes, phylogenetic grouping and antimicrobial resistance in *E. coli* strains isolated from free-ranging wild mammals from Santa Isabel do Rio Negro Region.

Animal species	Strain	Virulence genes														Phylogenetic group				Antimicrobial resistance[1]
		eae	bfp	stx1	stx2	papC	papEF	sfa	fyuA	iucD	hly	cnf1	cvaC	traT	malX	A	B1	B2	D	
rodent (*Mesomys hispidus*)	29								x					x		x				Amp
rodent (*Oecomys* sp.)	30								x							x				Amp, Amo
marsupial (*Metachirus* sp.)	31																			Tet
bat (*Molossus molossus*)	33	x							x						x				x	Susceptible
bat (*Molossus molossus*)	34															x				Amp, Amo
bat (*Molossus molossus*)	35														x	x				Amp, Amo
bat (*Molossus molossus*)	36								x			x						x		Amp, Amo
bat (*Glossophaga soricina*)	37/1								x			x			x		x			Amp
	37/2																	x		Amp
	37/3								x						x			x		Amp
	37/4								x						x		x			Susceptible
	37/5								x								x			Susceptible
bat (*Desmodontinae* sp.)	38/1	x							x					x	x	x				Susceptible
	38/2													x			x			Susceptible
	38/3													x		x				Susceptible
bat (*Mesophylla macconnelli*)	39															x				Amp, Amo
bat (*Tonatia saurophila*)	40								x						x				x	Amp, Amo
bat (*Lophostoma* sp.)	41/1								x			x							x	Amp
	41/2								x										x	Amp, Amo
bat (*Lophostoma* sp.)	42								x									x		Susceptible

[1]AMP: ampicillin, AMO: amoxicillin, TET: tetracycline.

Results and Discussion

Isolation and prevalence of VG and phylogenetic groups in *E. coli*

E. coli strains were isolated in 13 out of 14 (92%) and 13 out of 22 (59%) of the animals from CSP and SIRNR, respectively. The occurrence of *E. coli* in the mammalian gut can vary enormously, but typically *E. coli* makes up more than 90% of gut microbiota in humans and only 56% of gut microbiota in wild mammals (Gordon and Cowling, 2003; Tenaillon *et al.*, 2010). The recovery rate of *E. coli* was higher in animals from CSP, which may reflect a more intense exposure to human contamination of the environment. According to Gordon and Cowling (2003), the probability that a given host is carrying *E. coli* depends on the frequency of exposure, the likelihood that an exposure would result in the establishment of a population and, finally, the average period of time that an *E. coli* population could remain in a host. These issues are relevant when evaluating the importance of wildlife interactions with humans. Therefore, a total of 18 and 20 *E. coli* strains were successfully isolated from mammals in CSP and SIRNR, respectively. Characterizations of these strains are summarized in Tables 1 and 2.

This study verified that in the majority of *E. coli* isolates from CSP, both the number and range of VGs were higher than in those isolated from SIRNR; the same was true for antimicrobial resistance patterns. Whereas in SIRNR only two out of 20 isolated strains (10%) contained three or more VGs, in CSP seven out of 18 (39%) showed this profile. Considering that *E. coli* VGs are rare in the microbiota of wild animals but are not rare in humans (Tenaillon *et al.*, 2010) and that the domestication status (which can be defined as living in proximity with humans beings) of an animal host is the principal environmental force that shapes the genetic structures of commensal *E. coli* populations (Escobar-Páramo *et al.*, 2006), strains recovered from wild animals in CSP would reflect a broader anthropogenic action.

This hypothesis may be reinforced by the fact that the *eae* gene was only present in 10% (2/20) of strains in animals taken from SIRNR and in 28% (5/18) of those taken from CSP. However, none of the isolates were positive for either the *stx1* or *stx2* genes. The *eae+/stx-* markers are associated with the DEC, attaching and effacing of *E. coli,* and although strains with these markers are recognized as pathogens for humans and domestic animals, few studies have described their presence in wildlife (Bardiau *et al.*, 2010; Obwegeser *et al.*, 2012). Although a complete characterization of the *eae+* strains that were found in this survey has not yet been performed, a search for *bfpA* genes revealed one strain from CSP with the profile *eae+/bfpA+*. CSP is a natural area with a high anthropogenic impact, and isolates with this profile are linked to typical EPEC, which are strains that have been epidemiologically associated with human beings (Trabulsi *et al.*, 2002). It is important to acknowledge that the strain was isolated from a coati, specie that actively interacts with park visitors and their waste.

Furthermore, approximately 33% (6/18) of the strains isolated from CSP samples had some combination of the following genes: *papEF, cvaC, traT* and *malX.* All of these genes have been correlated with the pathogenic potential of ExPEC (Johnson *et al.*, 2001; Maynard *et al.*, 2004); however, only a single strain (5.5%) from SIRN exhibited similar findings (*malX* and *traT*). Previous studies have indicated that the presence of different combinations of VGs may provide a competitive advantage for the establishment of bacteria in intestinal microbiota as well as in the development of extra-intestinal infections (Diard *et al.*, 2010). P fimbria genes, such as *papC* and *papEF,* are important indicators of pathogenicity and have been associated with isolates that cause urosepsis in pets and humans (Maynard *et al.*, 2004). The *papEF* gene was present in 22% (4/18) of the strains isolated from CSP animals, but it was absent in SIRNR isolates. The *cvaC* gene is linked to ColV, a plasmid that occurs significantly more frequently in ExPEC recovered from neonatal meningitis compared to commensal *E. coli* (Johnson *et al.*, 2010; Bélanger *et al.*, 2011), and it was likewise found exclusively in the strains isolated from CSP mammals. The gene *traT* encodes a cell surface lipoprotein that increases bacterial resistance to the lytic actions of complement (Johnson and Stell, 2000). This gene was found in 67% (12/18) of CSP strains and only 20% (4/20) of SIRNR isolates.

The *malX* gene, a pathogenicity island (PAI) marker of the archetypal uroseptic strain CTF073 (06:K2:H1), is associated with urinary tract infections in both humans and pets, in addition to human pyelonephritis, bacteremia and neonatal meningitis (Johnson *et al.*, 2001; Johnson *et al.*, 2003). Surprisingly, this gene was found in 35% of SIRNR strains (7/20) and in 22% (4/18) of CSP strains. The discovery of this gene even in isolates from a less impacted environment may be due to the fitness demonstrated by strains possessing PAIs. As these genetic elements increase the competitive abilities of their carriers in the intestinal environment compared to isolates in which they are absent, they contribute to the intestinal persistence of *E. coli* (Östblom *et al.*, 2011; Diard *et al.*, 2010). Thus, if through it's gregarious behavior a wild animal acquires an *E. coli* strain that is harboring this gene, the presence of this marker could subsequently be amplified within the animal population. Out of all of the animals that were examined in this study, half of bats were found to harbor *E. coli malX+*. This is notable, as bats are characterized by living in large colonies and have the ability to travel through diverse ecological niches (Mühldorfer, 2013).

A phylogenetic classification indicated that 56% (10/18) of the strains isolated from CSP belonged to phylogenetic groups B2 and D, whereas in SIRNR this percentage was slightly lower (40%, 8/20). The majority of

commensal strains are derived from phylogenetic groups A and B1, whereas pathogenic strains generally belong to groups B2 and D. Group B2 is largely composed of ExPEC, which is a heterogeneous group of strains that are able to asymptotically colonize the intestines of several different hosts (Smith *et al.*, 2007). Most importantly, a B2 strain that contained eight of the ExPEC genes that were included in our study was only isolated from animals in CSP. These genes encoded for adhesins (*papC/papEF* and *sfa*), an iron capture system (*fyuA*), toxins (*hly* and *cnf1*), protectin (*traT*) and a ColV associated-virulence plasmid (*cvaC*) (Smith *et al.*, 2007; Bélanger *et al.*, 2011). Although the pathogenicity of these strains to wild animals is unknown, we can hypothesize that these animals were exposed to sources of potentially pathogenic bacteria because extra-intestinal VGs are rare in isolates from wildlife, as has been previously described (Tenaillon *et al.*, 2010).

Antimicrobial susceptibility

The presence of resistant bacteria in animals that had not been directly exposed to antimicrobials may reflect the degree of local anthropization (Costa *et al.*, 2013). A total of 83% (15/18) of the strains isolated from CSP and 65% of those isolated (13/20) from SIRNR demonstrated resistance to one or more antimicrobial agents. However, isolates from SIRNR were almost exclusively resistant to aminopenicillins, whereas strains from CSP were resistant to cephalosporins, sulfonamide, aminoglycoside, tetracycline and fluoroquinolone, in addition to presenting multidrug resistance (Table 1 and 2). Among the drugs tested, chloramphenicol was the only antimicrobial agent to which all of the strains that were recovered from CSP were susceptible. It is interesting to note elevated aminopenicillin resistance indices were found in SIRNR strains (12 out of 20 strains), ten of which were isolated from bats. These drugs are widely used in the treatment of human disease, as well as in companion and production animals (Maynard *et al.*, 2004), and their active compounds remain almost completely intact when they are eliminated into the environment. This may lead to environmental contamination and a dissemination of antimicrobial resistance (Costa *et al.*, 2013). Because SIRNR has fewer human and domestic animal residents, the results suggest that the observed resistance phenomenon was not limited to its original niches and that wildlife may have an important role in its spread. Bats are capable of traveling through wide geographic distances and diverse ecological niches; furthermore, their colonial population pattern facilitates the sharing of strains (Mühldorfer, 2013).

Salmonella isolation

Two strains of *Salmonella enterica,* serotype Belem and subspecies *diarizonae* serotype 60:r:e,n,z15, were respectively isolated from a bat and a rodent; both serotypes have been previously associated with reptiles (Schröter *et al.*, 2004; Riemann and Cliver, 2006). As they share the same terrestrial forest environment, it is not unexpected that serotypes are transmitted between rodent and reptile species. The Belem serotype that was isolated from a bat has been correlated with human infection in a previous survey that was performed in the Amazon region (Loureiro *et al.*, 2010). The involvement of free-ranging wildlife in the transmission and dissemination of *Salmonella* spp. to humans and other animals remains poorly understood, but a variety of *Salmonella* serotypes have been isolated from both healthy and sick bats (Mühldorfer, 2013). The geographic and ecological conditions that arise from the low levels of basic sanitation in the area promote the persistence of *Salmonella* spp. in the Amazon ecosystem (Loureiro *et al.*, 2010), and wildlife may contribute to its dissemination.

Conclusions

In conclusion, taking into account the different VG profiles and antimicrobial resistance patterns of *E. coli* strains isolated from individuals living in natural areas with dissimilar degrees of anthropization, the results obtained in this study indicate that human activity might increase the presence of resistant and/or potentially pathogenic *E. coli* strains in the environment. We also found that antimicrobial resistance is not limited to areas with greater anthropogenic impact. Regarding *Salmonella* spp., we observed that in natural environments, free-ranging wildlife might contribute to a cycle of perpetuation and dissemination of these bacterial species. Although the impact of these potential pathogens on wildlife is unknown, bacteria isolated from free-ranging wild animals may provide relevant information about environmental health and should therefore be more deeply studied.

Acknowledgments

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