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Detection and antimicrobial resistance of *Enterobacteriaceae* other than *Escherichia coli* in raccoons from the Madrid region of Spain

José Antonio Orden[1], Abel Martínez-Rodrigo[1,4], Ana Isabel Vela[2,3], José Francisco Fernández-Garayzábal[2,3], Clara Hurtado-Morillas[1], Alicia Mas[1]✉, Gustavo Domínguez-Bernal[1]

[1]INMIVET, Department of Animal Health, Faculty of Veterinary Science, [2]Department of Animal Health, Faculty of Veterinary Science, [3]VISAVET Health Surveillance Centre, [4]Department of Animal Science, Faculty of Veterinary Science, Complutense University of Madrid, 28040 Madrid, Spain
alimas@ucm.es

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Abstract

Introduction: Raccoons are an invasive alien species widely distributed in the Madrid region of Spain. These animals can carry a variety of enteric bacteria with associated antimicrobial resistance, which can infect humans and livestock. However, to our knowledge, the presence of non-*E. coli Enterobacteriaceae* in raccoons has not been previously studied. **Material and Methods:** We conducted a study to examine the species distribution of *Enterobacteriaceae* isolates other than *E. coli*, as well as their antimicrobial resistance, in the faeces of 83 raccoons in the Madrid region. **Results:** We detected 12 *Enterobacteriaceae* isolates other than *E. coli* belonging to seven different species: *Citrobacter freundii* (1 isolate), *Citrobacter gillenii* (3 isolates), *Citrobacter murliniae* (1 isolate), *Citrobacter portucalensis* (2 isolates), *Enterobacter hormaechei* subsp. *hoffmannii* (1 isolate), *Hafnia paralvei* (2 isolates) and *Raoultella ornithinolytica* (2 isolates). These isolates were found in 7 of the 83 (8.4%) animals studied. To our knowledge, this study is the first report of the presence of non-*E. coli Enterobacteriaceae* in raccoon faeces. All isolates but one were resistant to at least one of the 14 antimicrobials tested. Resistance to ampicillin (83.3%), amoxicillin-clavulanic acid (50%) and cefoxitin (33.3%) was the most frequent. **Conclusion:** Our study indicates that raccoons are a potential source of infection with *Enterobacteriaceae* other than *E. coli* for humans and livestock in the Madrid region.

Keywords: *Enterobacteriaceae* other than *E. coli*, raccoons, antimicrobial resistance, *Citrobacter* spp.

Introduction

The majority of *Enterobacteriaceae* genera besides *Escherichia* are usually opportunistic nosocomial pathogens and cause a wide spectrum of human infections (9). The status of antimicrobial resistance among *Enterobacteriaceae* other than *Escherichia* in wild animals is poorly understood because these genera are rarely isolated. In addition, studies testing for antimicrobial resistance in isolates of these genera from wild animals are scarce (5, 7, 10, 13).

Raccoons can carry a variety of enteric bacteria in their faeces, such as pathogenic and antimicrobial-resistant *E. coli*, which can infect humans and livestock and may represent a public health risk (14). However, to our knowledge, the presence of non-*E. coli Enterobacteriaceae* in raccoons has not been previously studied. Raccoons are an invasive alien species widely distributed in the Madrid region of Spain and live in close proximity to humans (6). As raccoons are considered both an ecological and a health risk (6, 14), the government of the Madrid region authorised a control programme that involved their capture, removal and euthanasia (14).

In a previous work, we studied the presence of zoonotic *E. coli* isolates and antimicrobial-resistant *E. coli* in faecal samples from raccoons in the Madrid region (14). These samples were used in this study with the aim of examining the species distribution of *Enterobacteriaceae* other than *E. coli* carried by the raccoons of the region and to investigate the presence of antimicrobial resistance in isolates of these species.

Material and Methods

The sites, trapping and sample collection used in this study have been described previously (6). Briefly, 83 faecal samples from apparently healthy raccoons (46 male and 37 female) were collected between October 2017 and March 2019. Trapping was carried out at nine sites in primarily periurban areas in the Madrid region (14). Following capture, the raccoons were weighed and then euthanised by veterinarians from the regional administration. Immediately after euthanasia, whole faecal samples were collected directly from the rectum, placed in sterile plastic bottles, and kept refrigerated until submitted to the laboratory the day after sampling. Faecal samples were plated on MacConkey agar and incubated overnight. After incubation, up to five colonies were selected from each sample. Isolates of *E. coli* and *Enterobacteriaceae* other than *E. coli* were initially differentiated by biochemical tests, including hydrogen sulphide, citrate, urease and indole. *Enterobacteriaceae* isolates other than *E. coli* were sub-cultured on Columbia agar overnight and identified by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with a Bruker MALDI Biotyper system (Bruker Daltonik, Bremen, Germany) (17) and sequencing of their complete 16S ribosomal RNA (rRNA) genes (21). The 16S rRNA sequences were compared with those of other Gram-negative species available in the GenBank database, using the EzTaxon server (http:/eztaxon-e.ezbiocloud.net/) (23).

Antimicrobial testing was performed using the disc-diffusion method and according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (3). The following 14 antimicrobials belonging to 6 different classes were tested: ampicillin, amoxicillin-clavulanic acid, cefoxitin and ceftriaxone (β-lactams); streptomycin, kanamycin, amikacin and gentamicin (aminoglycosides); tetracycline (tetracyclines); chloramphenicol (phenicols); sulphafurazole and trimethoprim-sulphamethoxazole (sulfonamides); and nalidixic acid and ciprofloxacin (quinolones). All antimicrobial susceptibility discs were provided by Oxoid (Basingstoke, UK). *Escherichia coli* ATCC 25922 was used as the control strain. The growth inhibition area of each isolate was measured and then each isolate was classified as susceptible, intermediate or resistant based on the breakpoints provided by the CLSI for *Enterobacteriaceae* (Table 1) (3).

Results

A total of 12 *Enterobacteriaceae* isolates other than *E. coli* were isolated from 8.4% of the animals studied (n=7). The isolates were identified as *Citrobacter freundii* (1 isolate), *C. gillenii* (3 isolates), *C. murliniae* (1 isolate), *C. portucalensis* (2 isolates), *Enterobacter hormaechei* subsp. *hoffmannii* (1 isolate), *Hafnia paralvei* (2 isolates) and *Raoultella ornithinolytica* (2 isolates), the *Citrobacter* genus being the most frequently identified (58.3%, 7/12). In our study, MALDI-TOF MS identification was successful only to the genus level and conclusive identification at the species level was possible only after sequencing the 16S rRNA gene (Table 2).

Table 1. Interpretive criteria for *Enterobacteriaceae* using disc diffusion susceptibility testing reported as inhibition zone diameters (mm)

Antimicrobial agent	Disc (μg)	Susceptible	Intermediate	Resistant
Ampicillin	10	≥17	14–16	≤13
Amoxicillin-clavulanic acid	30 (20/10)	≥18	14–17	≤13
Cefoxitin	30	≥18	15–17	≤14
Ceftriaxone	30	≥23	20–22	≤19
Streptomycin	10	≥15	12–14	≤11
Kanamycin	30	≥18	14–17	≤13
Amikacin	30	≥17	15–16	≤14
Gentamicin	10	≥15	13–14	≤12
Tetracycline	30	≥15	12–14	≤11
Chloramphenicol	30	≥18	13–17	≤12
Sulphafurazole	300	≥17	13–16	≤12
Trimethoprim-sulphamethoxazole	25 (1.25/23.75)	≥16	11–15	≤10
Nalidixic acid	30	≥19	14–18	≤13
Ciprofloxacin	5	≥26	22–25	≤21

The Bruker MALDI Biotyper system gives more than one probable identification result with different identification score values. Generally, the score values are higher in the first identification and go down in the following options. As can be seen in Table 2, the score values in the first identification option are higher than in the second one. In our experience, an identification based exclusively on the score values of the first identification option is not always the most accurate; it is helpful to also consider the results of the second identification option (17). For this reason, we evaluated the consistency of MALDI-TOF identification results taking into consideration the two best scores provided by the Bruker Biotyper in MALDI-TOF MS resolved to consistency categories A–D. Category A signifies that the correct species is the unique species with a score value ≥2.000; category B that the correct species is the first ranked but a different species is in the second rank also with a score value ≥2.000; C that the first and second matches have score values ≥2.000 but the correct species is the second ranked; and D that the first matches have score values >2.000 and the second matches have scores > or <2.000, but the correct species is neither the first nor second ranked. The isolates within the consistency category A are considered accurately identified, those within categories B and C are considered inconclusively identified and those within the D category are considered misidentified. Applying this criterion, no isolate was accurately identified, and most isolates were inconclusively elucidated as to their species or were misidentified (Table 2). All isolates but one were resistant to at least one antimicrobial, and almost half of the isolates (5/12) were resistant to three antimicrobials (Table 3). Resistance to ampicillin (83.3%, 10/12), amoxicillin-clavulanic acid (50%, 6/12) and cefoxitin (33.3%, 4/12) was the most frequent (Table 3).

Table 2. Identification by sequencing of the 16S ribosomal RNA (16S rRNA) gene and matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF MS) of 12 *Enterobacteriaceae* isolates other than *E. coli* from raccoons in the Madrid region of Spain

Isolate reference	Sequencing of the 16S rRNA gene		MALDI-TOF MS		
	Identification	Similarity (%)	First identification (score value) [a]	Second identification (score value) [a]	Consistency [b]
23147c	*Enterobacter hormaechei* subsp. *hoffmannii*	98.8	*Enterobacter cloacae* (2.296)	*Escherichia coli* (2.254)	D
23167b	*Citrobacter freundii*	98.8	*Citrobacter braakii* (2.458)	*Citrobacter freundii* (2.337)	C
23353b	*Citrobacter portucalensis*	99.3	*Citrobacter braakii* (2.467)	*Citrobacter freundii* (2.282)	D
23375a	*Citrobacter portucalensis*	99.5	*Citrobacter freundii* (2.289)	*Citrobacter braakii* (2.261)	D
23375c	*Raoultella ornithinolytica*	99.9	*Raoultella ornithinolytica* (2.399)	*Raoultella planticola* (2.301)	B
23380a	*Citrobacter murliniae*	99.2	*Citrobacter braakii* (2.397)	*Citrobacter freundii* (2.154)	D
23380b	*Hafnia paralvei*	99.1	*Hafnia alvei* (2.292)	*Dickeya chrysanthemi* (1.504)	D
23380c	*Hafnia paralvei*	99.1	*Hafnia alvei* (2.292)	*Dickeya chrysanthemi* (1.504)	D
23381a	*Raoultella ornithinolytica*	99.7	*Raoultella ornithinolytica* (2.383)	*Raoultella planticola* (2.245)	B
23381c	*Citrobacter gillenii*	99.0	*Citrobacter gillenii* (2.460)	*Citrobacter freundii* (2.122)	B
26650a	*Citrobacter gillenii*	99.5	*Citrobacter gillenii* (2.408)	*Citrobacter freundii* (2.140)	B
26650c	*Citrobacter gillenii*	99.5	*Citrobacter gillenii* (2.408)	*Citrobacter freundii* (2.140)	B

[a] – First and second identification best matches with their respective score values provided by the Biotyper identification list
[b] – Consistency ranking list of the first two best matches: B, the correct species is the first ranked but a different species is in the second rank also with a score value ≥ 2.000; C, the first and second matches have score values ≥2.000 but the correct species is the second ranked; D, the first matches have score values > 2.000 and the second matches have scores > or <2.000, but the correct species is neither the first nor second ranked

Table 3. Antimicrobial susceptibility of 12 *Enterobacteriaceae* isolates other than *E. coli* from raccoons in the Madrid region of Spain

Isolate reference	Identification	Susceptibility to:							
		AMP	AMC	FOX	ST	K	SF	NA	CIP
23147c	*Enterobacter hormaechei* subsp. *hoffmannii*	R	R	R	S	S	I	S	S
23167b	*Citrobacter freundii*	R	R	R	S	S	I	S	S
23353b	*Citrobacter portucalensis*	R	R	R	S	S	I	I	I
23375a	*Citrobacter portucalensis*	R	R	R	S	S	S	S	S
23375c	*Raoultella ornithinolytica*	R	S	S	R	S	S	R	S
23380a	*Citrobacter murliniae*	S	S	S	I	S	I	S	S
23380b	*Hafnia paralvei*	R	R	S	I	S	S	S	S
23380c	*Hafnia paralvei*	R	R	S	I	S	S	S	S
23381a	*Raoultella ornithinolytica*	R	S	S	S	S	I	S	I
23381c	*Citrobacter gillenii*	I	S	S	S	R	R	S	I
26650a	*Citrobacter gillenii*	R	S	S	S	S	I	S	S
26650c	*Citrobacter gillenii*	R	S	S	S	S	I	S	S

AMP – ampicillin; AMC – amoxicillin-clavulanic acid; FOX – cefoxitin; ST – streptomycin; K – kanamycin; SF – sulphafurazole; NA – nalidixic acid; CIP – ciprofloxacin; S – susceptible; I – intermediate; R – resistant. All isolates were susceptible to ceftriaxone, amikacin, gentamicin, tetracycline, chloramphenicol and trimethoprim-sulphamethoxazole

Discussion

To our knowledge, this is the first report analysing the presence of non-*E. coli Enterobacteriaceae* in raccoon faeces. Matrix-assisted laser desorption/ ionisation–time-of-flight mass spectrometry has been used before to identify non-*E. coli Enterobacteriacea*e (9, 11). The lower performance of the MALDI-TOF MS technique observed in our results can be explained by the omission of some of the species identified from the Biotyper database (for example, *H. paralvei* or *C. portucalensis*) or the difficulty in distinguishing different *Citrobacter* species due to the very similar spectra generated, which is congruent with the high similarity of their 16S rRNA gene sequences (2, 18). These results suggest that identification of non-*E. coli Enterobacteriaceae* obtained by 16S rRNA gene sequencing is more reliable than identification obtained by MALDI-TOF MS.

Citrobacter spp. are opportunistic human pathogens which can cause nosocomial infections, sporadic infections and outbreaks, with *C. freundii* being the most commonly isolated (12). In animals, *Citrobacter* spp. have been associated with septicaemia in several species (4, 10, 15). Categorised alongside these *Citrobacter* spp., *E. hormaechei*, *H. paralvei* and *R. ornithinolytica* are also considered opportunistic pathogens but are infrequent ones in both humans and animals (1, 8, 16, 19, 22). In animals, *R. ornithinolytica* has been associated with septicaemia in calves (16) and *E. hormaechei* with respiratory disease in cattle and sheep (19, 22). Regardless of the clinical importance of the species identified, the high frequency of antimicrobial resistance detected was significant. Our results agree with other studies that also found high levels of resistance to β-lactams in *Enterobacteriaceae* other than *E. coli* (5, 13), including the bacterial species identified in this study (1, 8, 10, 16, 19, 20, 22). The antimicrobial resistance found in most of the bacterial species isolated in this study is of concern, as the species can act as a reservoir for the spread of antimicrobial resistance to a particular preparation to other microbial inhabitants of the gut community of these animals or even other bacterial pathogens. Moreover, humans and livestock, mainly grazing cattle, sheep and goats, may become infected with antimicrobial-resistant *Enterobacteriaceae* other than *E. coli* after consuming food and water that has been contaminated with raccoon faeces.

In conclusion, our study shows that raccoons in the Madrid region of Spain harbour different species of *Enterobacteriaceae* other than *E. coli* which are considered opportunistic pathogens for humans and other animals. Therefore, it is recommended to monitor raccoons, as well as other feral animals that could interact with humans or other wild or domestic animals, for the presence of potentially pathogenic microorganisms and to investigate the levels of antimicrobial resistance in those microorganisms.

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