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Risk factors associated with faecal carriage of extended-spectrum cephalosporin-resistant *Escherichia coli* among dogs in Southeast Brazil

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ABSTRACT

Faecal carriage of extended-spectrum cephalosporin-resistant *Escherichia coli* (ESC-R *E. coli*) in dogs has been reported worldwide and can reduce the effectiveness of treatments against bacterial infections. However, the drivers that influence faecal carriage of ESC-R *E. coli* in dogs are poorly understood. The aims of this study were to estimate the prevalence of ESC-R *E. coli* among dogs prior to their admission to a veterinary teaching hospital and to identify risk factors associated with the faecal carriage of ESC-R *E. coli*. Rectal swabs (n = 130) were collected from dogs and screened for ESC-R *E. coli* using MacConkey agar supplemented with cefotaxime (2 μg/mL). *E. coli* species was confirmed by MALDI-TOF and screening of extended-spectrum beta-lactamase (ESBL) genes was conducted by multiplex PCR. Questionnaires were completed by each dog's owner to test several human and dog characteristics associated with ESC-R *E. coli*. The prevalence of faecal carriage of ESC-R *E. coli* was 9.2 % and 67 % of ESC-R *E. coli* isolates harboured ESBL genes including CTX-M alone or in combination with TEM. All ESC-R *E. coli* isolates were resistant to ceftriaxone, cefpodoxime, and cefotaxime and were susceptible to cefoxitin and carbapenems. The likelihood of carrying ESC-R *E. coli* was 15 times higher (OR = 14.41 [95 % CI: 1.80−38.02], $p < 0.01$) if the dog was treated with antibiotics 3–12 months prior to sampling and 8 times higher (OR = 7.96 [95 % CI: 2.96−92.07], $p < 0.01$) if the dog had direct contact with livestock, but 15 times lower (OR = 0.07 [95 % CI: 0.01−0.32], $p < 0.01$) if the dog was dewormed during the previous year. Our findings confirm the faecal carriage of ESC-R *E. coli* in subclinical dogs and call for further investigation regarding the impact of deworming on antibiotic-resistant bacteria in companion animals.

1. Introduction

Antimicrobial resistance (AMR) represents a major concern for human and animal health (Ventola, 2015). In order to reduce the burden of AMR, the World Health Organization included extended-spectrum cephalosporin-resistant (ESC-R) *Enterobacterales* as a 'global priority pathogen' due to the limited options available for effective antimicrobial treatment against their infections in both humans and animals (WHO, 2017). ESC-R *Enterobacterales,* particularly ESC-R *Escherichia coli*, have been isolated from livestock, wildlife, and companion animals, and have been positively correlated with extensive use of beta-lactam antibiotics in veterinary settings (Benavides et al., 2018; Chen et al., 2019). ESC-R *E. coli* can be found as either a commensal bacteria in the large intestine at equilibrium with the local microbiota or as pathogenic bacteria expressing virulence genes and causing intestinal diseases (Martens et al., 2018). In fact, several studies have reported ESC-R *E. coli* isolates from healthy and sick dogs and cats (Liakopoulos et al., 2018; Maeyama et al., 2018; Zhang et al., 2018). The presence of resistance

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genes in commensal *E. coli* in animals is a concern for the increase in AMR among pathogenic bacteria since resistance genes can be horizontally transferred between commensal and pathogenic strains (Loayza et al., 2020). Furthermore, commensal ESC-R *E. coli* in dogs could spread resistance within the community, particularly through faecal-oral contamination. However, the drivers underlying the acquisition and dissemination of ESC-R *E. coli* among dogs remain poorly understood.

Since the first reports of ESC-R *E. coli* among dogs from Japan in 1988 (Matsumoto et al., 1988) and Spain in 1998 (Teshager et al., 2000), ESC-R *E. coli* have been increasingly reported worldwide in dogs and cats (Jung et al., 2020; Kaspar et al., 2018; Okpara et al., 2018; Rusdi et al., 2018; Wedley et al., 2017). The ESC-R phenotype is often associated with the production of extended-spectrum beta-lactamases (ESBL) (Mughini-Gras et al., 2019). Extended-spectrum beta-lactamase genes have been globally reported in commensal and clinical ESC-R *E. coli* isolates obtained from companion animals (Bortolami et al., 2019; Hong et al., 2020; Karkaba et al., 2019; Ortega-Paredes et al., 2019; Pepin-Puget et al., 2020). However, the prevalence of faecal carriage of ESC-R *E. coli* in dogs (here referring to the number of colonized dogs over the total dogs sampled) differs within and between continents. For instance, the prevalence ranged from 1% (Canada) to 17 % (Mexico) in North America (Lefebvre et al., 2006; Rocha-Gracia et al., 2015), from 9% (Brazil) to 40 % (Ecuador) in South America (Carvalho et al., 2016; Ortega-Paredes et al., 2019), from 2% (Denmark) to 84.2 % (The Netherlands) in Europe (Baede et al., 2015; Damborg et al., 2015; Dupouy et al., 2019), from 14 % (China) to 81.8 % (Pakistan) in Asia (Abbas et al., 2019; Ho et al., 2011), from 17.5 % (Tunisia) to 94 % (Angola) in Africa (Albrechtova et al., 2014; Sallem et al., 2013), and from 7% (New Zealand) to 13 % (Australia) in Oceania (Karkaba et al., 2019; Rusdi et al., 2018). While these observations could be partially explained by differences in study methodologies and experimental designs, the biological factors underlying this variability have also been poorly explored.

Identifying the risk factors associated with the presence of ESC-R *E. coli* in dogs can contribute to the adoption of efficient strategies to limit its spread. In Europe, a limited number of studies have focused on the drivers of ESC-R *E. coli*, identifying the consumption of raw meat or poultry diets, history of hospitalization, and contact between dogs and livestock as increasing the probability of carrying ESC-R *E. coli* in dogs (Schmidt et al., 2015; van den Bunt et al., 2020; Wedley et al., 2017). However, to our knowledge, no study has been conducted in Latin America, where cephalosporins are widely used among veterinarians (Ortega-Paredes et al., 2019). The aims of this study were to estimate the prevalence of faecal carriage of ESC-R *E. coli* in subclinical dogs prior to their admission to a veterinary teaching hospital in Brazil and to identify the risk factors associated with the carriage of ESC-R *E. coli*.

2. Material and methods

2.1. Study area and sample size

The study was carried out at the Veterinary Hospital of the School of Veterinary Medicine and Animal Science at Sao Paulo State University (HV/FMVZ-UNESP) in the city of Botucatu. This city is located in the southeast region of Brazil and has an estimated population of 146,497 inhabitants. The population is predominantly urban and has a high Human Development Index (HDI) of 0.800 (IBGE, 2017). The dog population in Botucatu was estimated to be 27,735 dogs (Instituto Pasteur, 2018). We estimated our sample size to be 125 dogs based on an expected ESC-R *E. coli* prevalence of 9% among dogs in Brazil (Carvalho et al., 2016) with an acceptable margin of error of 5% and a desired confidence of 95 % using Epi Info 7.2.2.6 ™ (CDC, 2018).

2.2. Faecal sampling and questionnaires to identify risk factors

Between August and December 2018, faecal samples were obtained from 130 dogs seen at the referral Veterinary Hospital of the UNESP University prior to their admission (Salgado-Caxito et al., 2021). Dog ages ranged from 2 months to 10 years. Our inclusion criteria were: i) complete absence of clinical signs of infectious diseases assessed by the veterinarian collecting the sample and ii) absence of antimicrobial treatment at least three months prior to sampling. Rectal samples were collected from the first 130 dogs meeting the inclusion criteria before any procedure using sterile cotton swab. Samples were stored at 4 °C in Stuart transport medium (Oxoid, Hampshire, England) and processed within 24 h.

We administered a questionnaire to each dog's owner during faecal collection. The questionnaire included 13 questions related to the dog's characteristics, such as prior treatment (*i.e.,* antimicrobial therapies, vaccination, and deworming), contact with other animals or humans working with livestock, type of food ingested by the dog (including consumption of raw meat/poultry), antibiotic use by household (*i.e.,* humans and/or other pets), and owner's knowledge about antimicrobials. The variable 'owner's knowledge' was assessed by the owner's ability to identify bacteria as the target of antibiotics by completing the sentence 'antibiotics are used to treat diseases caused by …'. The variable 'vaccination' was assessed based on whether the owners had vaccinated their animals with the main vaccines available in this area (e.g., canine distemper, adenovirus-2, and canine parvovirus) in the previous 12 months. The 'deworming' variable referred to whether the owner administered any deworming drug to their dog within the previous 12 months. Questions are detailed in Supplementary Material 1. Data were entered using the free open source KoBoToolbox software (https://www.kobotoolbox.org/). All owners signed a consent form confirming their understanding and acceptance of the study objectives and their voluntary enrolment. This study was approved under protocol 0090/2018 by the Ethical Committee in Animal Use (CEUA) of the FMVZ - UNESP/Botucatu (registration number on CONCEA – National Council for Animal Control and Experimentation: CIAEP/CONCEA nº 01.0115.2014 – 05/06/2014).

2.3. ESC-R E. coli isolation

Faecal samples were streaked onto MacConkey agar (Oxoid Hampshire, England) supplemented with 2 μg/mL cefotaxime sodium salt (Sigma-Aldrich, St. Louis, Missouri, USA) to select for cefotaxime non-susceptible *E. coli* strains indicating ESBL/pAmpC production (CLSI, 2018). Plates were incubated in standard atmospheric conditions (100 kPa) for up to 48 h at 37 °C. An *E. coli* strain containing the $bla_{\text{CTX-M-15}}$ gene (donated by the Microbiology Laboratory of the IBB-UNESP/Botucatu) was used as a positive control. An *E. coli* isolate susceptible to beta-lactam antibiotics, obtained from a dog sample (donated by the Microbiology Laboratory at the HV/FMVZ-UNESP), which was susceptible to amoxicillin with clavulanic acid (30 μg), ceftriaxone (30 μg), ceftiofur (30 μg), ceftazidime (30 μg), cefovecin (30 μg), and cefquinome (10 μg), was used as a negative control. We selected and purified three presumptive *E. coli* colonies from positive plates based on their morphology. These isolates were first screened using biochemical tests according to the *Enterobacterales* identification test (EPM-MILi-Simmons' Citrate) developed by Ishiguro et al. (1978) and Toledo et al. (1982a, 1982b). Isolates were stored at UNESP, then shipped to Chile in transport medium and subsequently stored at −80 °C with 20 % glycerol. Isolates were further cultured on MacConkey agar supplemented with cefotaxime (2 μg/mL) for confirmation of species by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, BioMérieux, Marcy l'Etoile, France) at the Genomics and Resistant Microbes (GeRM) Group of the Millennium Initiative for Collaborative Research On Bacterial Resistance (MICROB-R), Santiago, Chile.

2.4. Antimicrobial susceptibility testing and ESC-R E. coli confirmation

Antimicrobial susceptibility was tested by performing the disk diffusion method according to the CLSI M100:28ED (CLSI, 2018). According to this CLSI document, cefpodoxime (10 μg) with inhibition zone ≤ 17 mm, ceftazidime (30 μg) with inhibition zone ≤ 22 mm, aztreonam (30 μg) with inhibition zone ≤ 27 mm, cefotaxime (30 μg) with inhibition zone ≤ 27 mm, and ceftriaxone (30 μg) with inhibition zone ≤ 25 mm of *E. coli* isolates may indicate ESBL production. Therefore, we tested these antibiotics in addition to cefoxitin (30 μg, breakpoints for resistant (R), intermediate (I), or susceptible (S) strain: R≤14 mm, I = 15−17 mm, and S≥18 mm) to confirm the extended-spectrum cephalosporin-resistance phenotype and to improve the sensitivity of ESBL detection. To assess co-resistance to carbapenems, we also tested imipenem (10 μg, R ≤ 19 mm, I = 20−22 mm, and S ≥ 23 mm), meropenem (10 μg, R ≤ 19 mm, I = 20−22 mm, and S ≥ 23 mm), and ertapenem (10 μg, R ≤ 18 mm, I = 19−21 mm, and S ≥ 22 mm). The *E. coli* ATCC25922 strain was used for quality control during each assay following the recommendations of CLSI M100:28ED, as well as the breakpoints (Supplementary Material 2).

2.5. Phenotypically confirmation of extended-spectrum beta-lactamase (ESBL) production and detection of ESBL genes

Extended-spectrum beta-lactamase production was confirmed in all ESC-R *E. coli* isolates using the double-disk synergy test (Benavides et al., 2018). Briefly, disks of ceftriaxone (30 μg), ceftazidime (30 μg), cefepime (30 μg), and aztreonam (30 μg) were used along with a disk of amoxicillin with clavulanic acid (30 μg) placed in the center of the plate at approximately 20 mm. The presence of an inhibition zone (ghost zone) around any of the cephalosporin disks towards the disk containing the clavulanic acid after 18−20 hours of incubation aerobically at 37 °C was considered positive for ESBL production.

The presence of the most common genes of ESBL enzymes (bla_{CTX-M}, bla_{SHV}, and bla_{TEM}) was tested using a multiplex PCR assay (Table 1). Briefly, each isolate was suspended in DNA/RNA free water and subjected to heating at 100 °C for 10 min, followed by centrifugation at 19,000 rcf for 2 min. After DNA extraction, 1 μL of the supernatant was added to 24 μL of the reaction mixture (5 μL of Buffer 5X, 1 μL of $MgCl_2$ (25 mM), 0.5 μL of dNTPs (10 mM), 1 μL of each forward primer (10μM), 1 μL of each reverse primer (10 μM), 0.125 μL of Taq (5U/μL), and 11.375 μL of DNA/RNA free water). PCR conditions were as follows: i) initial denaturalization at 95 °C for 2 min; ii) 30 cycles to denaturation, annealation, and extension at 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, respectively; and iii) final extension at 72 °C for 5 min. Amplicons obtained were analysed using 1.5 % agarose gel electrophoresis and visualized by UV trans-illuminator. The *E. coli* strain SCL-1290 containing the three ESBL genes: bla_{CTX-M}, bla_{SHV}, and bla_{TEM} (donated by the GeRM Group of MICROB-R - Chile) was used as a positive control.

2.6. Statistical analysis

The prevalence of ESC-R *E. coli*, defined as the recovery of at least one ESC-R *E. coli* per dog over the total dogs sampled, was reported. Confidence intervals (95 % CI) were calculated using the *binom.confint* function (Agresti-Coull method) in the *binom* package in R 3.6.1 (R Core Team, 2015). Using a logistic regression, we evaluated the association between faecal carriage of ESC-R *E. coli* and several factors related to the characteristics of the dogs, owners, and households. The binary nature of our response variable (*i.e.,* resistant or not) required using generalized linear models (GLM) with binomial errors with the *glm* function in R. First, a statistical model including all explanatory variables was built with no interaction terms (AIC = 80.6). The statistical significance of each variable ($p < 0.05$) was assessed by the Wald's test. We also built the same model adding either an interaction between the variables 'dog's deworming' and 'treatment in the last year' (AIC = 81.2) or the variables 'deworming' and 'hospitalization' (AIC = 81). However, these interactions increased the model's AIC and were not statistically significant ($p > 0.05$) in the full model. Thus, they were not included in the final model. Second, we built a model including only significant variables ($p < 0.05$) from the full model, which was selected over the full model based on a lower AIC (AIC = 64) and was therefore used to calculate the final odds ratio confidence intervals for the statistically significant variables.

3. Results

3.1. Characteristics of the dogs included in the study

The sampled dogs (61 %, 70/130) were located in houses in Botucatu or in cities within a 350Km radius from Botucatu (39 %). The dogs sampled included 50.8 % (66/130) adults (from one to seven years old), 16.1 % (21/130) puppies (dogs less than one year old), and 33.1 % (43/130) seniors (dogs above seven years old). Purebred dogs represented 55.4 % (72/130) of the study population and 56.2 % (73/130) were female. Although we did not have the information for 6 dogs, the type of food ingested by the dogs was mainly kibble (95.2 %, 118/124), but owners also provided raw (8.1 %, 10/124) and cooked meat/poultry (63 %, 78/124) along with vegetables (46 %, 57/124). The features of the studied dog population are detailed in Table 2.

3.2. Prevalence and risk factors associated with ESC-R E. coli in dogs based on questionnaire data

The prevalence ESC-R *E. coli* in dogs, defined as the recovery of at least one ESC-R *E. coli* per dog over the total sampled dogs, was 9.2 % (12 out of 130 dogs) [95 % CI: 0.05–15.57 %]. Statistical analysis was performed using data from 124 completed questionnaires (Table 2). In addition to the variables related to the characteristics of the dogs (age, gender, breed), we included answers from 13 questions in the logistic regression analysis (Supplementary Material 1). Variables with more than two alternatives (e.g., options 'Never used', 'More than 12 months', 'Between 6 and 12 months ago', 'Between 3 and 6 months ago', 'No answer' to the question regarding 'previous use of antimicrobials') were transformed into binomial variables according to biological criteria in order to maintain statistical power to detect significant effects by testing several categorical variables simultaneously.

Dogs treated with antibiotics 3–12 months prior to sampling were 15 times more likely to harbour ESC-R *E. coli* (GLM, Odds Ratio (OR) = 14.41 [95 % CI: 1.80−38.02], $p < 0.01$) (Table 3). Likewise, dogs that

Table 1
Sequences, melting temperature, and amplicon size of primers used for detection of CTX-M-type, SHV-type, and TEM-type genes.

ESBL gene type	Primer name	Sequence (5′-3′)	Optimal melting temperature (°C)	Amplicon size (bp)	Reference
CTX-M-type	CTX-M-F_544bp	SCSATGTGCAGYACCAGTAA	55	544	(Jena et al., 2017)
	CTX-M-R_544bp	CCGCRATATGRTTGGTGGTG			(Jena et al., 2017)
SHV-type	SHV-F_795bp	TTATCTCCCTGTTAGCCACC		795	(Jena et al., 2017)
	SHV-R_795bp	GATTTGCTGATTTCGCTCGG			(Jena et al., 2017)
TEM-type	TEM-F_1074bp	GAAGACGAAAGGGCCTCGTG		1074	(Taşli and Bahar, 2005)
	TEM-R_1074bp	GGTCTGACAGTTACCAATGC			(Taşli and Bahar, 2005)

Table 2
Summary of answers extracted from the owner's questionnaires (n = 124).

Variable[1]	Response (n = total of dogs)	ESC-R *E. coli* carriage[2]		ESC-R *E. coli* non-carriage[3]	
		N	%	N	%
Age	Puppies (n = 20)	1	0.8%	19	15.3%
	Adults (n = 65)	7	5.6%	58	46.8%
	Seniors (n = 39)	4	3.2%	35	28.2%
Gender	Male (n = 54)	6	4.8%	48	38.7%
	Female (n = 70)	6	4.8%	64	51.6%
Breed	Mixed (n = 52)	8	6.5%	44	35.5%
	Purebred (n = 72)	4	3.2%	68	54.8%
Antibiotics used on dog during the previous year	Yes (n = 22)	5	4.0%	17	13.7%
	No/never (n = 89)	5	4.0%	84	67.7%
	Not answered (n = 13)	2	1.6%	11	8.9%
Other pets on household	Yes (n = 89)	9	7.3%	80	64.5%
	No (n = 35)	3	2.4%	32	25.8%
Antibiotic used on other pets in the household during the previous year	Yes (n = 42)	4	3.2%	38	30.6%
	No (n = 81)	8	6.5%	73	58.9%
	Not answered (n = 1)	0	0.0%	1	0.8%
Owner's knowledge about antibiotics[4]	Yes (n = 54)	5	4.0%	49	39.5%
	No (n = 64)	7	5.6%	57	46.0%
	Not answered (n = 6)	0	0.0%	6	4.8%
Dog's vaccination during the previous year[5]	Yes (n = 51)	2	1.6%	49	39.5%
	No (n = 68)	10	8.1 %	58	46.8%
	Not answered (n = 5)	0	0.0%	5	4.0%
Dog's deworming during the previous year[6]	Yes (n = 84)	4	3.2%	80	64.5%
	No (n = 34)	8	6.5%	26	21.0%
	Not answered (n = 6)	0	0.0%	6	4.8%
Dog's hospitalization during the previous year	Yes (n = 8)	2	1.6%	6	4.8%
	No (n = 113)	10	8.1 %	103	83.1%
	Not answered (n = 3)	0	0.0%	3	2.4%
Dog's access to the street	Yes (n = 90)	9	7.3%	81	65.3%
	No (n = 32)	3	2.4%	29	23.4%
	Not answered (n = 2)	0	0.0%	2	1.6%
Dog's contact with livestock	Yes (n = 29)	6	4.8%	23	18.5%
	No (n = 91)	6	4.8%	85	68.5%
	Not answered (n = 4)	0	0.0%	4	3.2%
Household working with livestock	Yes (n = 22)	3	2.4%	19	15.3%
	No (n = 97)	9	7.3%	88	71.0%
	Not answered (n = 5)	0	0.0%	5	4.0%
Antibiotic used on humans in the household during the previous year	Yes (n = 71)	7	5.6%	64	51.6%
	No (n = 30)	4	3.2%	26	21.0%
	Not answered (n = 23)	1	0.8%	22	17.7%
Habitat	Urban (n = 107)	8	6.5%	99	79.8%
	Rural (n = 17)	4	3.2%	13	10.5%
Raw meat/poultry ingested by the dog	Yes (n = 10)	0	0%	10	8.1 %
	No (n = 112)	12	9.7%	100	80.6%
	Not answered (n = 2)	0	0%	2	1.6%

[1] Qualitative answers extracted from the owner's questionnaires.
[2] Absolute and percentual number of owners of colonized dogs in each category.
[3] Absolute and percentual number of owners of non-colonized dogs in each category.
[4] "owner's knowledge about antibiotics" was assessed by the owner's ability to identify bacteria as the target of antibiotics completing the sentence 'Antibiotics are used to treat diseases caused by …'.
[5] "vaccination" was assessed as whether owners vaccinated their animals with the main vaccines available in this area (canine distemper, adenovirus-2, and canine parvovirus) (Tizard, 2020) in the previous 12 months by answering the question 'Was your dog vaccinated for canine distemper, adenovirus-2, and parvovirus in the previous year by a veterinarian?
[6] "deworming" variable refers to whether the owner administrated any deworming drug to their dog within the previous 12 month by answering the question 'Was your dog dewormed during the previous year?'.

had direct contact with livestock were 8 times more likely to harbour ESC-R *E. coli* (GLM, OR = 7.96 [95 % CI: 2.96−92.07], $p < 0.01$) even when controlling for the household location (urban or rural) and a household member working with livestock. In contrast, dogs that were dewormed during the previous year were 15 times less likely to harbour ESC-R *E. coli* (GLM, OR = 0.07 [95 % CI: 0.01−0.32], $p < 0.01$).

We did not find a statistically significant association between the presence of ESC-R *E. coli* and several other explanatory variables (GLM, $p > 0.05$), including the dog's individual characteristics (age, gender or breed), owner's knowledge about antibiotics, dog's hospitalization during the previous year, dog's access to the street, dog's vaccination status, dog's intake of raw meat/poultry, whether humans in the household used antibiotics or worked with livestock, the presence of other pets in the household, or the type of habitat (urban or rural).

3.3. Antimicrobial resistance profiles and ESBL gene detection

We recovered and characterized 35 isolates of ESC-R *E. coli* from the 12 positive dogs, all of which were positive for ESBL production based on the double-disk synergy test (Supplementary Material 2). To avoid duplicating identical strains, isolates from the same sample showing the same antimicrobial resistance pattern and ESBL gene were excluded from further analysis. Therefore, we selected 18 isolates from the 12 dogs. Susceptibility testing showed that these 18 isolates were resistant to ceftriaxone, cefpodoxime, and cefotaxime (Table 4). In addition, 56 % (10/18) of the isolates were resistant to aztreonam. Resistance to ceftazidime was observed in 11 % (2/18) of the isolates. Resistance to cefoxitin and carbapenems was not observed. There were three different cephalosporins and aztreonam resistance profiles among the isolates: i) ceftriaxone, cefpodoxime, and cefotaxime (8 isolates), ii) ceftriaxone, cefpodoxime, cefotaxime, and aztreonam (8 isolates), and iii) ceftriaxone, cefpodoxime, cefotaxime, ceftazidime, and aztreonam (2 isolates).

ESBL genes were detected in 12/18 (67 %) of the ESC-R *E. coli* isolates recovered from eight dogs. CTX-M-type was identified in 12 isolates and the combination CTX-M and TEM was detected in three isolates from two dogs. SHV was not detected. In addition, in 6 of the isolates, the ESBL type could not be detected with our scheme (Table 4).

4. Discussion

ESC-R *E. coli* circulating among dogs has been increasingly reported

Table 3
General Linear Model regression to explain the presence of ESC-R *E. coli* in dogs extracted from the owner's questionnaires (n = 124). Reduced Model (AIC = 64) including only significant variables (p < 0.05) from the Full Model (AIC = 80.60) that included all explanatory variables.

Variables	Odds Ratio [95 % CI]	p-value[1]
REDUCED MODEL (AIC = 64)[2]		
(Intercept)	0.09 [0.02−0.24]	<0.01
Antibiotics used on dog during the previous year (yes)	**14.41 [1.80−38.02]**	**<0.01**
Dog's contact with livestock (yes)	**7.96 [2.96−92.07]**	**<0.01**
Dog's deworming during the previous year (yes)[3]	**0.07 [0.01−0.32]**	**<0.01**
FULL MODEL (AIC = 80.6)		
(Intercept)	0.01 [0.00−0.72]	0.07
Age (years)	1.04 [0.72−1.49]	0.84
Gender (male)	2.26 [0.34−16.54]	0.39
Breed (mixed)	0.29 [0.02−2.81]	0.30
Antibiotics used on dog during the previous year (yes)	**42.48 [2.86−1109.21]**	**0.01**
Dog's contact with livestock (yes)	**28.92 [3.09−799.86]**	**0.01**
Household working with livestock (yes)	4.26 [0.18−101.38]	0.34
Dog's hospitalization during the previous year (yes)	2.7 [0.06−64.36]	0.55
Owner's knowledge about antibiotics (yes)[4]	1.54 [0.21−12.89]	0.67
Dog's access to the street (yes)	13.23 [0.85−838.32]	0.13
Dog's vaccination during the previous year (yes)[5]	0.36 [0.02−4.05]	0.42
Dog's deworming during the previous year (yes)[3]	**0.01 [0.00−0.16]**	**<0.01**
Antibiotic used on humans in the household during the previous year (yes)	0.22 [0.03−1.69]	0.15
Habitat (urban)	0.48 [0.02−13.69]	0.64
Other pets on household (yes)	11.36 [0.83−422.15]	0.11
Antibiotic used on other pets in the household during the previous year (yes)	0.25 [0.01−3.56]	0.34
Raw meat/poultry ingested by dogs (yes)	0.3 [0.00−16.83]	0.61

[1] Values for p < 0.05 were considered statistically significant (variables in **bold**).
[2] The Reduced model was used to calculate the final odds ratio confidence intervals for the statistically significant variables.
[3] "deworming" variable refers to whether the owner administrated any deworming drug to their dog within the previous 12 month by answering the question 'Was your dog dewormed during the previous year?'.
[4] "owner's knowledge about antibiotics" was assessed by the owner's ability to identify bacteria as the target of antibiotics completing the sentence 'Antibiotics are used to treat diseases caused by …'.
[5] "vaccination" was assessed as whether owners vaccinated their animals with the main vaccines available in this area (canine distemper, adenovirus-2, and canine parvovirus) (Tizard, 2020) in the previous 12 months by answering the question 'Was your dog vaccinated for canine distemper, adenovirus-2, and parvovirus in the previous year by a veterinarian?'.

worldwide, but the factors associated with its faecal carriage remain poorly understood. Here, we report a prevalence of 9.2 % of faecal carriage of ESC-R *E. coli* in subclinical dogs, which is within the range of prevalence (9 %–40 %) reported in previous studies in Latin America (Carvalho et al., 2016; Melo et al., 2018; Ortega-Paredes et al., 2019; Rocha-Gracia et al., 2015). Analyses of questionnaires revealed that a dog's prior treatment with antibiotics (3–12 months before sampling) and direct contact with livestock increased the likelihood of faecal carriage of ESC-R *E. coli,* whereas a dog's previous deworming decreased the likelihood.

The likelihood of faecal carriage of ESC-R *E. coli* was 15 times higher when dogs were submitted to antibiotic therapy 3–12 months prior to sampling (OR = 14.41 [95 % CI: 1.80−38.02], p < 0.01). Several studies have described the selection pressure of antimicrobial usage as an important risk factor for the appearance and persistence of resistant *E. coli* in animals, including ESC-R *E. coli* (Buckland et al., 2016; Burow et al., 2014; Chang et al., 2015; Chantziaras et al., 2014; Okpara et al., 2018; Schmidt et al., 2018; Wedley et al., 2017, 2017). To date, Brazil has not reported antimicrobial usage in animals nor prescription patterns among veterinarians to the authors' knowledge. However, according to the Brazilian Compendium of Veterinary Products (https://sistemas.sindan.org.br/cpvs/), there are 163 veterinary products containing antimicrobial compounds approved specifically for dogs and no resolutions to regulate the purchase of antimicrobials for animals in Brazil, facilitating misuse. Our study also suggests that carriage of ESC-R *E. coli* could last for several months following treatment or the dogs could have reacquired ESC-R *E. coli* from other (unknown) sources since positive dogs did not receive antimicrobials in the three months prior to our sampling. This calls for further longitudinal studies investigating the duration of ESC-R *E. coli* carriage among dogs.

Table 4
Resistance profile of isolates and extended-spectrum beta-lactamases genes identified in 18 ESC-R *E. coli* isolates from 12 dogs.

Dog ID[1]	Isolate[2]	ESBL[3]	Antimicrobial resistance profile[4]
1	MS1_270	ND	CRO CPD CTX ATM
	MS1_271	ND	CRO CPD CTX
2	MS1_274	ND	CRO CPD CTX
3	MS1_276	CTX-M/TEM	CRO CPD CTX ATM
	MS1_278	CTX-M/TEM	CRO CPD CTX CAZ ATM
4	MS1_279	ND	CRO CPD CTX
5	MS1_282	ND	CRO CPD CTX
	MS1_283	CTX-M	CRO CPD CTX ATM
6	MS1_285	CTX-M	CRO CPD CTX ATM
7	MS1_287	CTX-M	CRO CPD CTX
8	MS1_290	CTX-M	CRO CPD CTX
	MS1_292	CTX-M	CRO CPD CTX ATM
9	MS1_293	CTX-M	CRO CPD CTX ATM
10	MS1_296	CTX-M	CRO CPD CTX ATM
	MS1_298	CTX-M/ TEM	CRO CPD CTX
11	MS1_299	ND	CRO CPD CTX
12	MS1_302	CTX-M	CRO CPD CTX ATM
	MS1_304	CTX-M	CRO CPD CTX CAZ ATM

Abbreviations: CRO-ceftriaxone, CPD-cefpodoxime, CTX-cefotaxime, CAZ-ceftazidime, ATM-aztreonam, ND-not detected ESBL genes in the molecular analysis by multiplex PCR.
[1] Dog number assigned.
[2] ID designed for a given isolate.
[3] Type of extended-spectrum beta-lactamases enzymes.
[4] Antimicrobial susceptibility patterns as described in methods.

Dog's contact with livestock (*i.e.,* cattle, swine, equine, poultry) also increased the likelihood of ESC-R *E. coli* carriage by 8 times (OR = 7.96 [95 % CI: 2.96−92.07], p < 0.01), supporting similar findings in dogs from Germany (van den Bunt et al., 2020). The association with livestock was statistically significant even when controlling for the household location (urban or rural) and a household member working with livestock. This suggests that living in a rural area or in a household with farmers is not sufficient to increase the likelihood of ESC-R *E. coli* in dogs. ESC-R *E. coli* is widely disseminated among livestock in Brazil and antimicrobial usage in Brazilian herds may be extensive (Silva and Lincopan, 2012; Tomazi and dos Santos, 2020). Our study suggests that ESC-R *E. coli* could be circulating between livestock and dogs. This could be tested by simultaneously sampling and molecular characterization of *E. coli* isolates and resistance genes in both populations.

To our knowledge, this is the first study to report that deworming in the previous year reduces the likelihood of faecal carriage of ESC-R *E. coli* in dogs (OR = 0.07 [95 % CI: 0.01–0.32], p < 0.01). Previous studies have demonstrated interactions between intestinal parasites and intestinal microbiota, including competition for resources in the host (Bucková et al., 2018; Newbold et al., 2017; Walk et al., 2010). For instance, increasing dysbiosis has been observed in the microbiota of birds with a high burden of helminths (Newbold et al., 2017). Also, higher parasite levels have been correlated with high bacterial diversity in mammals (Kreisinger et al., 2015; Rubel et al., 2020). Interestingly,

one of these studies showed an association between multiple parasite detection and enrichment of gut microbiota bacterial gene pathways involved in beta-lactam resistance and cationic antimicrobial peptide resistance in agropastoral farmers (Rubel et al., 2020). Among the alterations in the intestinal microbiota composition caused by endoparasites, helminths are known to produce antimicrobial peptides (Su et al., 2018; Zhang and Hou, 2013). Thus, the association between ESC-R *E. coli* and endoparasites may result from both dysbiosis and selection by the antimicrobial peptides produced. In addition, enteric pathogens could influence the expression of resistance genes. A previous study demonstrated that *Giardia* sp could induce virulence gene expression in *E. coli* and alter commensal isolates to become opportunistic pathogens (Gerbaba et al., 2015). Another study showed that an association between deworming in pig farms and a lower prevalence of *Campylobacter* sp resistance to tetracycline resulted from an overall improvement of hygiene practices and antimicrobial stewardship (Schuppers et al., 2005). Although our results did not establish causality, our findings call for further research exploring whether enteric pathogens can modulate the carriage of antibiotic-resistant bacteria.

Microbiological analyses showed three phenotypic profiles of cephalosporin resistance in which all ESC-R *E. coli* were resistant to ceftriaxone, cefotaxime, and cefpodoxime (Table 4). A similar study in Rio de Janeiro reported much lower resistance (<30 %) to these three antibiotics in ESC-R *E. coli* isolates (Carvalho et al., 2016), while another study described 100 % resistance to ceftazidime, cefotaxime, and cefpodoxime among ESBL/AmpC producing *E. coli* isolates from healthy dogs (Aslantaş and Yilmaz, 2017). Differences in the prevalence of resistance to cephalosporins between studies might reflect the variety of molecular mechanisms that can generate ESC-R *E. coli* including efflux pumps, pore deficiencies, expression of different ESBL genes or other beta-lactamases, and mutations in the chromosomal AmpC gene (Baede et al., 2015). None of the ESC-R *E. coli* isolates found in this study were resistant to carbapenems, suggesting a low prevalence of carbapenemase producers among *E. coli* from dogs in Botucatu. However, despite the very restricted use of carbapenems in the veterinarian sector of Brazil, we did not screen for carbapenem resistance directly from faecal samples and this prevalence could be underestimated (Reynolds et al., 2019).

All ESC-R *E. coli* isolates were positive based on the double-disk synergy test and 67 % carried CTX-M-type alone or in combination with TEM. In the current study, the prevalence of faecal carriage of ESBL *E. coli* in dogs was 6.1 %, which is lower than other studies conducted in healthy dogs in Brazil (9 %–31 %) (Carvalho et al., 2016; Melo et al., 2018). The presence of ESBL genes was not detected in ESC-R *E. coli* from four dogs, requiring further investigation into other less prevalent genes and mechanisms responsible for the observed phenotypic resistance (e.g., PER, OXA, or AmpC genes).

Several limitations of this study could be addressed by future research. For example, information on the dog's treatment was obtained directly from the owners' answers to the questionnaire and were not assessed from medical records. Future work could compare the two sources of information to identify potential biases in owner responses. Furthermore, other factors that may influence ESC-R *E. coli* could not be tested without compromising the statistical power to detect potential effects due to the small sample size. Thus, the influence of these other factors, such as the presence of other pets in the household or different types of exposure opportunities (e.g., access to the street) could be included in future studies with larger sample sizes. Likewise, the lack of statistically significant associations between ESC-R *E. coli* and several other variables tested in this study could be due to a small sample size preventing us from detecting significant effects. Finally, screening of ESC-R *E. coli* was assessed based on whether bacteria grew on the selective medium without performing *E. coli* counts. This information could help to identify the factors that will influence both the carriage of ESC-R *E. coli* and its successful maintenance and excretion within the dog's intestinal microbiota.

5. Conclusion

Our study confirms the faecal carriage of ESC-R *E. coli* among dogs in Southeast Brazil, possibly reflecting a high (but unknown) misuse of antimicrobials among companion animals. The circulation of ESC-R *E. coli* among dogs indicates the need to establish pet surveillance programs and antimicrobial stewardship among veterinarians to reduce treatment failure of bacterial infections among dogs and potential transmission to humans and other animals. This study suggests the potential for transmission of ESC-R *E. coli* between companion animals and livestock in rural areas, which should be assessed by further studies in both populations. Our results also call for a better understanding of the interaction between gut parasites and antibiotic-resistant *Enterobacterales*.

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Declaration of Competing Interest

The authors of this paper have no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prevetmed.2021.105316.

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