Comparison of Antimicrobial-Resistant *Escherichia coli* Isolates from Urban Raccoons and Domestic Dogs

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ABSTRACT Wildlife can be exposed to antimicrobial-resistant bacteria (ARB) via multiple pathways. Spatial overlap with domestic animals is a prominent exposure pathway. However, most studies of wildlife-domestic animal interfaces have focused on livestock and little is known about the wildlife-companion animal interface. Here, we investigated the prevalence and phylogenetic relatedness of extended-spectrum cephalosporin-resistant (ESC-R) *Escherichia coli* from raccoons (*Procyon lotor*) and domestic dogs (*Canis lupus familiaris*) in the metropolitan area of Chicago, IL, USA. To assess the potential importance of spatial overlap with dogs, we explored whether raccoons sampled at public parks (i.e., parks where people and dogs could enter) differed in prevalence and phylogenetic relatedness of ESC-R *E. coli* to raccoons sampled at private parks (i.e., parks where people and dogs could not enter). Raccoons had a significantly higher prevalence of ESC-R *E. coli* (56.9%) than dogs (16.5%). However, the richness of ESC-R *E. coli* did not vary by host species. Further, core single-nucleotide polymorphism (SNP)-based phylogenetic analyses revealed that isolates did not cluster by host species, and in some cases displayed a high degree of similarity (i.e., differed by less than 20 core SNPs). Spatial overlap analyses revealed that ESC-R *E. coli* were more likely to be isolated from raccoons at public parks than raccoons at private parks, but only for parks located in suburban areas of Chicago, not urban areas. That said, ESC-R *E. coli* isolated from raccoons did not genetically cluster by park of origin. Our findings suggest that domestic dogs and urban/suburban raccoons can have a diverse range of ARB, some of which display a high degree of genetic relatedness (i.e., differ by less than 20 core SNPs). Given the differences in prevalence, domestic dogs are unlikely to be an important source of exposure for mesocarnivores in urbanized areas.

IMPORTANCE Antimicrobial-resistant bacteria (ARB) have been detected in numerous wildlife species across the globe, which may have important implications for human and animal health. Wildlife can be exposed to ARB via numerous pathways, including via spatial overlap with domestic animals. However, the interface with domestic animals has mostly been explored for livestock and little is known about the interface between wild animals and companion animals. Our work suggests that urban and suburban wildlife can have similar ARB to local domestic dogs, but local dogs are unlikely to be a direct source of exposure for urban-adapted wildlife. This finding is important because it underscores the need to incorporate wildlife into antimicrobial

resistance surveillance efforts, and to investigate whether certain urban wildlife species could act as additional epidemiological pathways of exposure for companion animals, and indirectly for humans.

KEYWORDS cephalosporin, dog, *Escherichia coli*, interface, phylogenetic, raccoon, urban

Human encroachment into natural habitats, urbanization, and wildlife adaptation to human activity have increased the extent to which humans and domestic animals interface with wildlife. Greater contact between humans, domestic animals, and wildlife increases the risk of infectious agent spillover (1–4). Our understanding of this phenomenon has mostly been driven by pathogen spillover from wildlife into human or domestic animal populations (e.g., Ebola virus, avian influenza virus, SARS-CoV, and SARS-CoV-2) (2, 5, 6). However, infectious agents can also spill over from human sources into wild animal populations through the environment, which can threaten public and domestic animal health if wildlife cause further spread and spillback into the human and/or domestic animal populations (1).

A quintessential example of spillover from human sources into wildlife is the dissemination of antimicrobial-resistant bacteria (ARB) (7–9). ARB that are typically associated with clinical settings have been detected in numerous wildlife species across the globe (9, 10). In general, wild animals are more likely to shed ARB if they are closer to human-dominated areas, such as livestock facilities, urban areas, landfills, and fish farms (8, 10–12). In some human-dominated settings, ARB prevalence in wildlife can be as high as 50% or more, such as in some bird, mesocarnivore, rodent, and ungulate populations (13–17). Further, wildlife present in these human-dominated areas tend to have ARB that are similar to those of local human and/or domestic animal populations, both in terms of genetic relatedness and the antimicrobial-resistance gene (ARG) profiles (18–20). Thus, it has become clear that many ARB detected in wildlife are of anthropogenic origin (10, 12). Further, because ARG can be horizontally transferred between bacteria via processes such as conjugation, there is a concern that AMR has the potential to spread in wildlife bacterial communities (21). Under this scenario, wildlife would not only act as vectors of AMR, but also as reservoirs (22, 23).

In urban settings, ARB have been detected in multiple wildlife species (e.g., rodent, gull, song bird species) (14, 23–25) and, in most cases, prevalence tends to be higher than in nonurban wildlife (14, 26). Urban wildlife can be exposed to ARB and associated ARG via multiple pathways, including contaminated waters, garbage or other food sources (9, 12, 23), and livestock manure (27). While ARB are unlikely to be directly transmitted between humans and wildlife, transmission could occur more readily via domestic animals. Companion animals are especially likely to be important because they frequently use the same green spaces as urban wildlife (28–30) and share several infectious agents with wildlife and humans (e.g., Hendra virus, *Salmonella* spp.) (31, 32), including ARB (32–34). Despite this potential risk, AMR research at the wildlife-companion animal interface has been explored infrequently and with conflicting results. In some cases, companion animals and wildlife have similar AMR profiles (15, 35), while in others there is less evidence of similarity (36), indicating that more research is needed.

Here, we compared ARB isolated from raccoons (*Procyon lotor*) and domestic dogs (*Canis lupus familiaris*) sampled in the metropolitan area of Chicago, IL, USA. We focused on raccoons and domestic dogs because they both frequently use urban green spaces (e.g., parks and backyards) (37), share several infectious agents (e.g., *Leptospira* spp., canine distemper virus), and can shed ARB (13, 34, 38). Further, our previous research revealed that raccoon and dog samples pooled by animal species had several ARG in common (39). In the present study, we explore the interface between raccoons and dogs in more detail by investigating the prevalence and phylogenetic relatedness of extended-spectrum cephalosporin-resistant *Escherichia coli* (ESC-R *E.*

coli) in 211 raccoons and 176 domestic dogs. ESC-R *E. coli* include both extended-spectrum beta-lactamase (ESBL) and AmpC beta-lactamase-producing *E. coli*, which are resistant to third generation cephalosporins (e.g., cefotaxime, ceftazidime). We focused on ESC-R *E. coli* because they are of increasing concern in human and veterinary medicine (40–43), and have been reported in healthy human (44–46), livestock (47, 48), and companion animal populations (41, 49–51), as well as in the environment (52–54). ESC-R *E. coli* has also been isolated from the feces of over 30 wildlife species (e.g., gulls, wild boar, mallard duck, rodent species) (8, 55), including over half of the 211 raccoons previously sampled in our system (13).

The specific objectives of this study were to (i) explore the extent to which raccoons and dogs have similar ESC-R *E. coli* profiles in terms of prevalence, phylogenetic relatedness, and number and types of ARG, and (ii) determine whether raccoons differed in ESC-R *E. coli* profile based on whether they were sampled at public parks (i.e., parks where people and dogs could enter) or at private parks (i.e., parks where people and dogs could not enter), and how this compared to domestic dogs. We hypothesized that raccoons would have a lower prevalence and diversity of ESC-R *E. coli* than dogs because of antimicrobial use in dogs, their intimate contact with humans (33, 34), and because of wildlife-domestic animal findings in other urban systems (e.g., reference 27). Additionally, we expected raccoons at public parks to have a higher prevalence of ESC-R *E. coli* than raccoons at private parks because of potentially higher contact rate with dog feces and human garbage. By extension, we also expected raccoons at private parks to have ESC-R *E. coli* that were more phylogenetically distinct to ESC-R *E. coli* isolated from dogs and raccoons at public parks.

RESULTS

Raccoon and domestic dog characteristics. Raccoons and dogs were sampled over the course of four seasons, from February to November 2018 in northwestern Chicago, IL, USA. Raccoons were captured and sampled from seven sites that differed based on whether they were urban or suburban and whether they were on private or public land (Fig. 1). Together, the seven sites covered a distance of ~40 km. At public sites, most dogs were required to be leashed by law. At private sites, dogs were not allowed to enter. Of the 211 raccoons sampled (17 of which were captured twice and one three times), 61.6% were sampled in suburban areas and 38.4% in urban areas, and 63.5% were sampled at public sites and 36.5% at private sites.

Domestic dogs were sampled at three of the seven sites where raccoons were sampled (two suburban and one urban) or at nearby dog parks (Fig. 1). Of the 176 dogs sampled, 12.5% were sampled from the same household as at least one other sampled dog. Based on dog owner survey results, 36.4% of dogs were ≤2 years of age, 42.6% were between 2 and 7, 19.3% were older than 7, and 1.7% had no age data. Stratified by sex, 56.3% dogs were males, 37.5% were females, and 6.2% had no data. In terms of antibiotic use, 30.1% of sampled dogs were on some form of antibiotic in the 12-months prior to sampling, 53.4% were not, 11.9% of owners were unsure, and 4.6% of owners did not respond. Based on where dogs were sampled, 48.3% of dogs were sampled at sites where raccoons were sampled and 51.7% at local dog parks. Most sampled dogs lived in the northwestern portion of the Chicago area (based on home ZIP code) (Fig. 1), and 64% of dogs had their home ZIP code that overlapped with at least one of the sites where raccoons were sampled (Fig. 1).

Domestic dogs had a lower prevalence of ESC-R *E. coli* than raccoons, but ESC-R *E. coli* bacteria isolated from dogs and raccoons were not genetically distinct and in some cases displayed a high degree of similarity and had multiple ARG in common. With a sample prevalence of 56.9% (95% confidence interval [CI] = 50.1% to 63.4%) and 16.5% (95% CI = 11.7% to 22.7%) for raccoons and dogs, respectively (Fig. 2A), there was a significantly higher odds of recovering at least one ESC-R *E. coli* isolate from raccoons than from dogs (Fisher's exact test; odds ratio [OR] = 3.44, 95% CI = 2.16 to 5.63; $P < 0.0001$). Whole-genome sequencing and multilocus sequence typing (MLST) revealed that of the 152 ESC-R *E. coli* isolates recovered from raccoons and
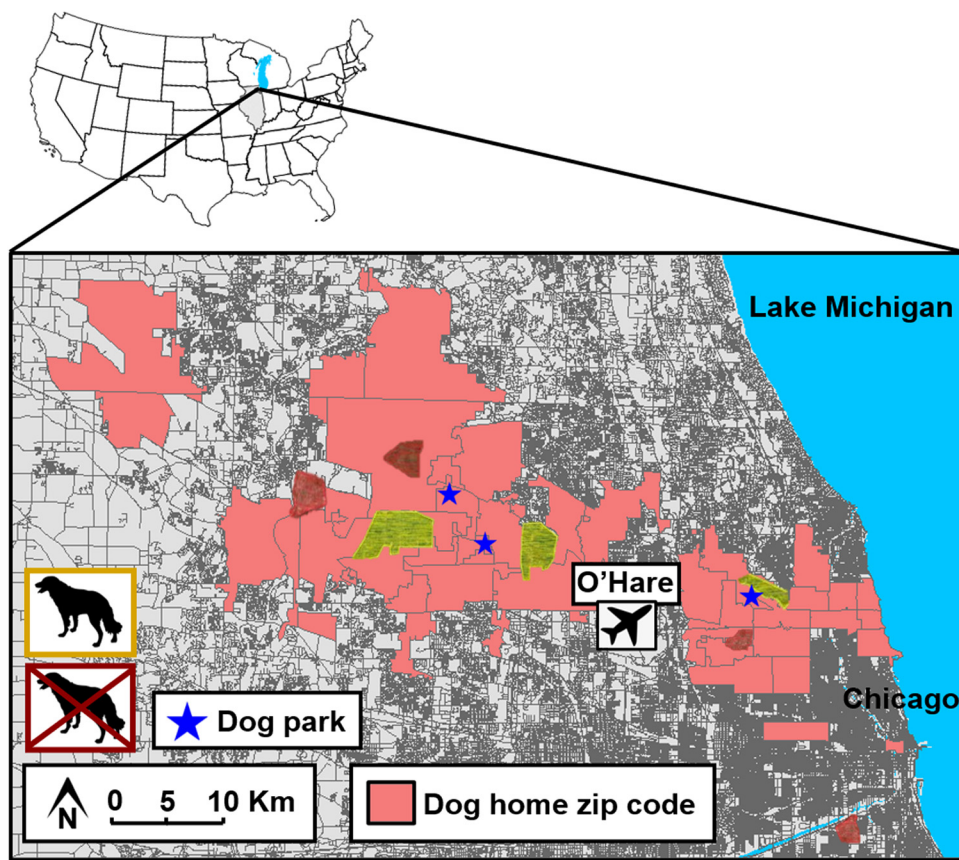
FIG 1 Sampling sites in the northwestern portion of the Chicago metropolitan area. Small dark red and yellow polygons depict sites where raccoons were sampled. The four small dark red polygons are private sites (i.e., sites where people and domestic dogs were not allowed to enter) and the three yellow polygons are public sites (i.e., sites where people and dogs were allowed to enter). Blue stars represent dog parks and pink polygons are dog home ZIP codes. Four shapefiles were used to create the map: (i) a street shapefile for Cook County (https://hub-cookcountyil.opendata.arcgis.com/datasets/4569d77e6d004c0ea5fada54640189cf_5), (ii) a street shapefile for DuPage County (https://gisdata-dupage.opendata.arcgis.com/datasets/roadtypecenterline?geometry=-89.010%2C41.659%2C-87.158%2C42.017), (iii) a Lake Michigan shapefile (https://gis-michigan.opendata.arcgis.com/datasets/5e2911231fe246128d0ff8495935ee85_12), and (iv) a U.S. shapefile (https://hub.arcgis.com/datasets/1b02c87f62d24508970dc1a6df80c98e_0?geometry=118.842%2C29.346%2C-4.029%2C67.392).

dogs (123 from raccoons and 29 from dogs), raccoons had a total of 55 unique sequence types (STs) and one unknown (the unknown ST closely resembled ST155, with variation in the *gyrB* allele only) and dogs had 20 unique STs and two unknown (one of the unknowns closely resembled ST58, with variation in the *parA* allele only, and the other was dissimilar to all STs) (Fig. 2B). Accounting for differences in samples sizes, bootstrapping the raccoon sample size to the dog sample size (i.e., from $n = 123$ to $n = 29$) revealed that the raccoon and dog populations likely shed a similar richness of STs (95% CI for raccoons = 16.1 to 23.8 using 1,000 bootstrap replicates). Of the STs detected, ST38 was most commonly detected in raccoon samples (8.8%), followed by ST973 (7.3%), and both ST68 and ST162 (4.8%) (Fig. 2B). For dogs, ST68 was most common (13.8%), followed by ST297 (10.3%) (Fig. 2B).

In terms of phylogenetic similarity, raccoons and dogs had 12 STs in common, including ST10, ST38, ST68, and ST131 (Fig. 2B). Core single-nucleotide polymorphism (SNP)-based phylogenetic analyses revealed that within-species average core SNP differences were similar to between-species average core SNP differences (raccoon to raccoon: 455.8 mean core SNP difference; dog to dog: 489.2; raccoon to dog: 480). Further, the maximum likelihood phylogenetic tree showed no clustering by species, with dog and raccoon samples randomly interspersed throughout the tree (Fig. 2C), which was supported
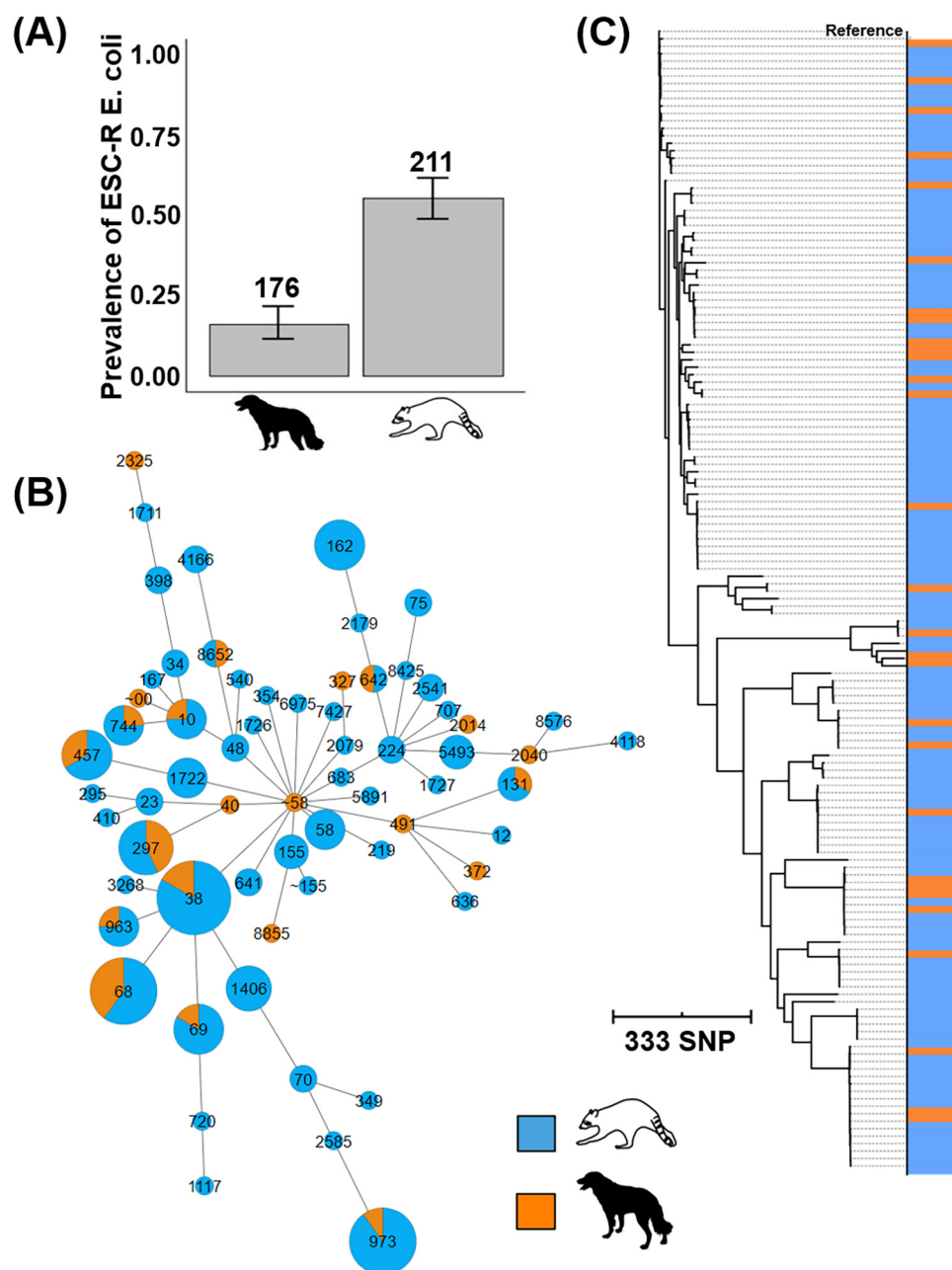
FIG 2 Prevalence and phylogenetic associations of ESC-R *E. coli* isolated from raccoons and domestic dogs. (A) Prevalence of ESC-R *E. coli*. Whiskers represent 95% confidence intervals and numbers above whiskers are sample sizes. (B) Minimum spanning tree of ESC-R *E. coli* sequence types (STs) detected in raccoons (blue) and domestic dogs (orange). The size of nodes represents the number of isolates and the length of lines connecting nodes represents the number of allelic differences. ST numbers preceded by a tilde were unknown. (C) Core SNP-based maximum likelihood phylogenetic tree of the 152 ESC-R *E. coli* and heatmap of isolates classified based on host species (i.e., raccoon, blue; dog, orange). The reference is *E. coli* K-12 strain MG1655.

by a lack of significant difference in the phylogenetic distance of ESC-R *E. coli* isolates by animal species (permutational multivariate analysis of variance [PERMANOVA]: $F_{1, 151}$ = 0.45, $P = 0.85$). Focusing on isolates that belonged to one of the 12 STs shared between raccoons and dogs (19 isolates from dogs and 51 isolates from raccoons), pairs of isolates displayed a high degree of similarity, as they differed by less than 20 core SNPs in all cases and were similar both within and between animal species (Fig. 3).

With regard to ARG, a total of 56 and 40 ARGs were identified in ESC-R *E. coli* isolated from raccoons and dogs, respectively, and most were found in isolates of both species
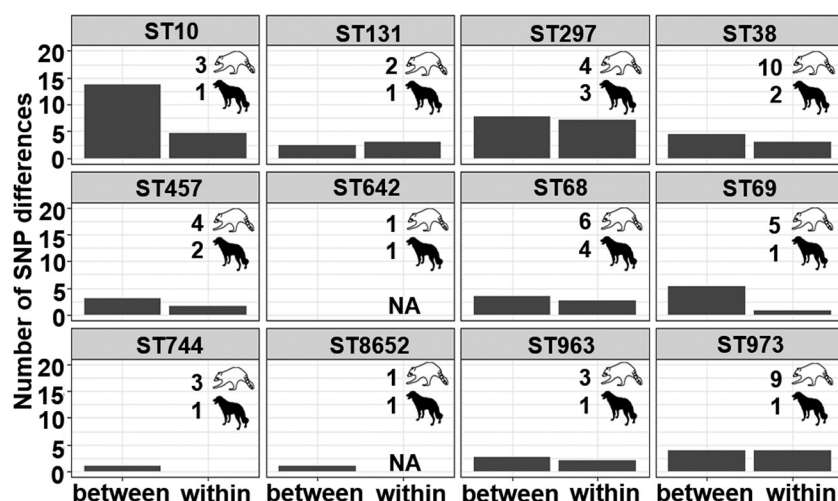
FIG 3 Mean number of core SNP differences between pairs of ESC-R *E. coli* isolates by sequence type (ST) based on whether pairs of isolates were from different animal species ("between") or the same animal species ("within"). Numbers next to raccoon and dog silhouettes are the number of isolates belonging to each animal species by ST. NA indicates that no comparison could be done.

(Fig. 4). Focusing on beta-lactam genes (i.e., *bla* genes), bla_{CMY-2} was the most prevalent in both raccoon and dog ESC-R *E. coli* isolates (54% and 62%, respectively), followed by bla_{TEM-1B} (26% and 21%, respectively). Further, 43.9% of beta-lactam genes detected in ESC-R *E. coli* isolated from raccoons were of bla_{CTX-M}-type, of which $bla_{CTX-M-15}$ was the most common, followed by $bla_{CTX-M-14}$ and $bla_{CTX-M-55}$. For dogs, bla_{CTX-M}-type genes accounted for 25% of beta-lactam genes, of which $bla_{CTX-M-1}$ and $bla_{CTX-M-55}$ were the most common. For non-beta-lactam genes, a greater proportion of ESC-R *E. coli* isolated from raccoons had fluoroquinolone and tetracycline ARGs than ESC-R *E. coli* isolated from dogs (Fig. 4).

Probability of isolating ESC-R *E. coli* from raccoons sampled at public parks was higher than for raccoons sampled at private parks, but only at suburban parks. After controlling for seasonal and urban-suburban context effects based on findings from previous work (13), binomial generalized linear mixed models (GLMMs) revealed that the odds of isolating ESC-R *E. coli* from raccoons varied significantly based on whether raccoons were sampled at public or private sites, with an interaction effect between whether a site was private or public and urban or suburban. Specifically, the odds of isolating ESC-R *E. coli* from raccoons was higher at public compared to private sites, but only in suburban sites and not urban sites (Table 1; Fig. 5).

ESC-R *E. coli* bacteria isolated from raccoons sampled at public parks were not phylogenetically distinct from those isolated from raccoons sampled at private parks or from those isolated from domestic dogs. There was no significant difference in the phylogenetic distance of ESC-R *E. coli* isolates recovered from raccoons sampled at public parks and raccoons sampled at private parks or from those recovered from domestic dogs (PERMANOVA: $F_{2,151} = 0.43$, $P = 0.95$).

DISCUSSION

Wildlife can be exposed to ARB via multiple pathways, including through spatial overlap with domestic animals. However, in this study, we found no evidence that spatial overlap with domestic dogs acts as a major source of exposure for urban-adapted raccoons. ESC-R *E. coli* were three times more likely to be recovered from raccoons than domestic dogs, although isolates obtained from raccoons were not genetically distinct from those obtained from dogs and in some cases displayed a high degree of similarity (i.e., differed by less than 20 core SNPs). When exploring the importance of raccoon spatial overlap with dogs and people at parks, we found that the odds of isolating ESC-R *E. coli* from raccoons was higher when raccoons were sampled at public
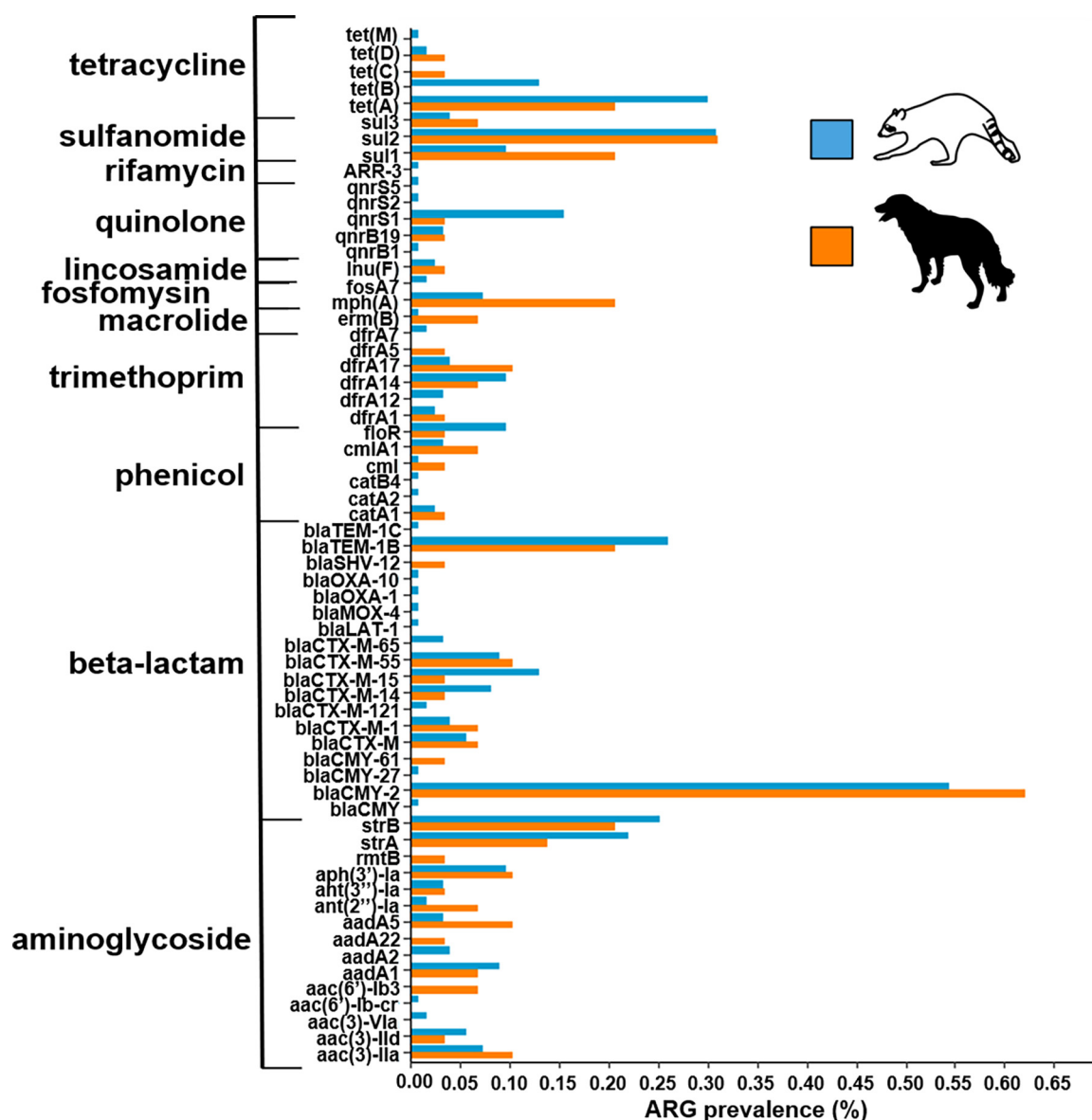
FIG 4 Prevalence of antimicrobial resistance genes (ARG) in ESC-R *E. coli* isolated from raccoons (blue) (*n* = 123) and domestic dogs (orange) (*n* = 29).

than at private parks, with this difference being only apparent at suburban and not urban parks. In terms of genetic relatedness of ESC-R *E. coli*, we found that ESC-R *E. coli* bacteria isolated from raccoons sampled at public parks were not distinct from those isolated from raccoons sampled at private parks or from those isolated from dogs.

It was surprising to find that raccoons had a higher prevalence of ESC-R *E. coli* than dogs, since dogs are considered reservoirs for AMR due to the use of antimicrobials in these animals and their close contact with humans and other animals in which antimicrobials are used (34). That said, wildlife could have higher AMR prevalence than dogs if they were exposed to ARB and ARG through pathways that dogs were less likely to be exposed to. For example, lakes and rivers are important pathways for the dissemination of ARB into the environment (52, 56, 57), and water-associated wildlife species are especially likely to be exposed (26, 58, 59). Raccoons select habitats with water bodies (60, 61) because a large proportion of their food is in or along rivers and lakes (62). Thus, it is possible that raccoons had a higher prevalence of ESC-R *E. coli* compared to

TABLE 1 Generalized linear mixed model results for isolating at least one ESC-R *E. coli* from raccoons[a]

Predictor variable	Odds ratio	95% CI	P
season (spring)	**8.05**	**(2.71–23.9)**	**<0.001**
season (summer)	**5.05**	**(2.03–12.59)**	**0.001**
season (winter)	0.49	(0.2–1.17)	0.11
urban-suburban context (urban)	**34.95**	**(5.42–225.39)**	**<0.001**
dog presence (yes)	**5.36**	**(1.26–22.83)**	**0.02**
urban context (urban) × dog presence (yes)	**0.07**	**(0.01–0.79)**	**0.03**

[a]Significant terms are depicted in boldface type (with 95% CI not overlapping with 1 and *P* < 0.05).

dogs because they were exposed to ARB via contaminated water sources. This is, however, speculative as no environmental samples were collected as part of this study. While previous work has suggested that differences in the prevalence of certain ARB between animals species could be attributed to differences in the host gut hospitability to certain bacteria (8, 55, 63), it is unlikely to be of importance here because ESC-R *E. coli* have previously been isolated from dogs in both clinical and community settings (64, 65). Further, work comparing the AMR profiles of owned and stray dogs and three mesocarnivore species supports the notion that environmental factors are more likely to be important than physiological ones (39). As such, differences in exposure risk are likely a more plausible explanation for the prevalence differences detected here than differences in host physiological characteristics. Differences in exposure risk may also explain differences in prevalence observed between urban and suburban raccoons, which are possibly due to variation in home range size and food availability, as discussed in reference 13.

While raccoons tended to have a higher sample prevalence of ESC-R *E. coli* than dogs, raccoons sampled at public parks were more likely to have ESC-R *E. coli* than raccoons sampled at private parks, but only in suburban areas and not urban areas. Previous work has shown the presence of domestic animals to be an important determinant of isolating ARB from wildlife (e.g., reference 66). However, since the dog population tended to have a low prevalence of ESC-R *E. coli* (16.5%), the presence of dogs themselves is unlikely to be the main factor associated with the differences detected at suburban sites. Instead, the difference in the number of people (with and without dogs) and the anthropogenic waste left at parks was potentially more influential. While water bodies are predicted to be the primary pathway of wildlife exposure to ARB, anthropogenic waste is also thought to be important (9). For example, wildlife can have a higher prevalence of ARB and ARG when using landfills (67), and similar ARB to those detected in landfills (59) or other wildlife sampled at landfills (68). Raccoons are
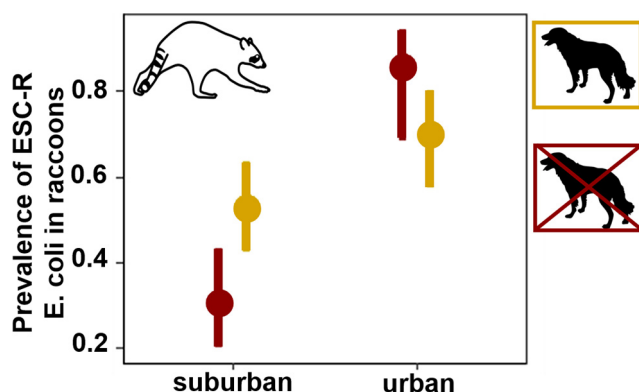

FIG 5 Raw prevalence of ESC-R *E. coli* in raccoons by urban-suburban context and dog presence (yellow, public park [i.e., people and domestic dogs can enter], red, private parks [i.e., people and domestic dogs cannot enter]). Whiskers are 95% confidence intervals. Raccoons were sampled in two public (*n* = 78) and two private (*n* = 52) suburban parks and one public (*n* = 56) and two private (*n* = 25) urban parks.

generalist and opportunistic feeders (69), and in urban and suburban areas they will feed on anthropogenic waste present in parks, either on the ground or in trash cans (70). Thus, raccoons sampled at public suburban parks may have had a higher prevalence than raccoons sampled at private suburban parks because of the higher exposure to people and anthropogenic waste. A lack of difference detected at urban parks could be because raccoons at both private and public parks were equally likely to be exposed to anthropogenic waste. However, because a small number of parks were examined (and only one urban public park), more work is needed to ascertain the importance of people and anthropogenic waste in influencing the prevalence of ARB in urban-adapted wildlife.

While dog and raccoon populations differed in ESC-R *E. coli* prevalence, ESC-R *E. coli* isolated from the two animal species were not genetically distinct. Further, in some cases raccoon and dog isolates differed by less than 20 core SNPs. Such a high degree of similarity could reflect transmission among dogs and raccoons sampled (71, 72). However, as well as having STs in common with dogs, raccoons also had several STs that were not detected in dogs and are typically associated with human sources, such as ST23, ST224, ST410, and ST167 (41). Further, ARB identified in wildlife have previously been attributed to human sources (18, 73), especially in urban areas (14, 25). This conforms to the general consensus that humans tend to play a more important role in the circulation of ARB and ARG in the community and the environment than companion animals (47). Hence, raccoons may have acquired ESC-R *E. coli* through exposure to human-derived sources of AMR rather than through contact with dog feces. Nevertheless, other human-associated STs, such as ST131 and ST10 (41), were found in both raccoons and dogs. Companion animals and people can have several ESC-R *E. coli* in common (74), either because of direct transmission or parallel microevolution (48). Thus, it is possible that dogs and raccoons had similar ESC-R *E. coli* because individuals of both species were exposed to human-associated AMR via different pathways. While the ESC-R *E. coli* isolated from raccoons could not be compared to those of people living in Chicago, work in other systems (e.g., reference 62) suggests that comparing the AMR profile of urban wildlife and coexisting human populations would be an important next step to take.

Finding no genetic distinction between ESC-R *E. coli* bacteria isolated from raccoons at public parks, raccoons at private parks, and dogs could indicate that raccoons and dogs have closely related ESC-R *E. coli* bacteria, or it could indicate that raccoons and dogs of Chicago present a diverse pool of ESC-R *E. coli* strains. Given that several ESC ARG tend to be transmitted horizontally via plasmids (42), we suspect the latter explanation is most likely. The fact that highly related ESC-R *E. coli* (i.e., differing by <20 core SNPs) were found between raccoons at private parks, raccoons at public parks, and dogs reinforces this point, and suggests that these bacteria are potentially being randomly disseminated to different hosts in the same environment. However, no firm conclusions can be made, partly because our study was limited by the number of isolates per sample (one per sample) and per host group (e.g., 29 for dogs versus 123 for raccoons). Given the number of ESC-R *E. coli* likely present per gram feces, examining a total of 152 isolates probably provided insufficient power to discern the diversity of ESC-R *E. coli* present in raccoons and dogs, and thus the degree of genetic relatedness. Further, other types of ARB and/or microbiology techniques may have provided better resolution for comparing AMR between raccoons and dogs. ESC-R *E. coli* were chosen because of their ease and frequency of isolation, and relevance to human medicine. Use of other ARB (e.g., methicillin-resistant *Staphylococcus aureus*) may or may not yield better resolution. Similarly, comparing the range of the resistance level between raccoon and dog samples using MICs may have provided more insight on the distribution of ARB in these two host species and should be explored in future studies. Thus, this study should be viewed as a first step toward understanding the ecology of AMR at the wildlife-companion animal interface.

In conclusion, an important finding of this study was the difference in prevalence of ESC-R *E. coli* between dogs and raccoons. We were over three times more likely to

recover ESC-R *E. coli* from raccoons than dogs. Raccoons have the potential to pose a risk to dogs if dogs come into contact with raccoon feces at parks or when raccoons visit residential backyards, especially if raccoon densities are high. Exploring whether AMR risk for dogs increases when dogs reside in areas where raccoons occur at high densities and have high prevalence of ARB would be a useful next step. Further, given the likely role of the environment for raccoon exposure to ARB, an important next step for studying AMR in companion animals would be to explore the importance of not only wildlife but also the environment. In previous work, we found that raccoons sampled in urban areas had a higher risk of exposure than raccoons sampled in suburban areas (13). Exploring whether similar patterns hold true for dogs while accounting for relevant epidemiological factors (e.g., dog diet, attendance at dog day care) (75, 76) would be insightful. In this way, we advocate that future work explore multiple AMR exposure pathways simultaneously (i.e., humans, domestic animals, wildlife, and the environment).

Environmental and wildlife AMR research has been grossly overlooked in understanding the epidemiology of ARB (9, 77), and our study highlights the need for continued research on wildlife AMR. To date, much wildlife AMR research has advocated targeting avian species (in particular gulls) as sentinels for AMR in the environment. We argue that mammalian species that reside in close proximity to humans, such as raccoons, could also be important targets. The fact that raccoons spend a large proportion of time in residential areas and along rivers and lakes (60, 78) makes them especially useful for understanding the spread and maintenance of ARB in urban and suburban environments. Since raccoons in many regions across the United States are tested for pathogens such as rabies virus, testing for the presence of clinically relevant ARB in feces and storing isolates for future genomics work would be a productive surveillance measure to initiate.

MATERIALS AND METHODS

Study site and design. In February to November 2018, raccoons were captured from seven sites in northwestern Chicago, IL, USA, of which four were suburban and three were urban (Fig. 1). Sites were classified as urban if the site and surrounding area (i.e., ~1 km buffer around each site) were composed of ≥80% impervious surface. Otherwise, sites were classified as suburban (for details see reference 13). Out of the seven sites, three were public sites (i.e., open to the public and domestic dogs) (Fig. 1), and four were private sites (i.e., inaccessible to the public and domestic dogs) (Fig. 1). Domestic dogs were sampled at each of the three public sites and at dog parks (park in which dogs mingle off leash) that were closest to three of the public sites (Fig. 1).

Raccoon and dog sampling. Raccoons were captured using box traps (Model 108, Tomahawk Live Trap Co., Tomahawk, WI, USA) (78) and immobilized with an injection of Telazol (Fort Dodge Animal Health, Fort Dodge, Iowa). Fecal samples were collected opportunistically from the rectum of each immobilized raccoon. After recovering from immobilization, all raccoons were released at the capture locations. Captures were approved by the University of Minnesota's Institutional Animal Care and Use Committee (protocol ID 1709-35105A) and by the Illinois Department of Natural Resources (permit number IDNR W17.0122).

Dogs were selected at random, but dogs less than 6 months of age were excluded. For every dog sampled, a standardized survey (Table S1 in the supplemental material) was given to dog owners detailing the age and sex of each dog, as well as history of antibiotic use in the past year. Dog owners were also asked for their home ZIP code. Dog fecal samples were collected by dog owners using their own dog waste bags or bags were provided by investigators. All dog and raccoon fecal samples were stored in brain heart infusion broth and 20% glycerol at −80°C until further analyses.

Phenotypic characterization of ESC-R *E. coli*. Presence of ESC-R *E. coli* was explored by testing *E. coli* susceptibility to cefotaxime, a third-generation cephalosporin. A detailed description of this procedure can be found in reference 13. Briefly, samples were enriched overnight in lauryl tryptose phosphate broth (Difco Laboratories, Detroit, MI, USA) and streaked onto CHROMagar ECC containing 2 μg/ml of cefotaxime (36, 79). If blue colonies (representative of *E. coli*) were obtained, one per sample was selected at random and restreaked on CHROMagar ECC containing 2 μg/ml of cefotaxime. All isolates were stored at −80°C until sequencing.

Sequencing, bioinformatics, and phylogenetic analyses. Whole-genome sequencing (WGS) was performed on all recovered ESC-R *E. coli* isolates. Details on DNA extraction, WGS, and quality check of raw reads can be found in reference 13.

Genetic associations among isolates were explored by first determining the multilocus sequence type (MLST) of each isolate. To do this, trimmed reads were assembled using SPAdes assembler (version 3.0) (80) with default parameters. The quality of assemblies was assessed by examining the N_{50} score of each isolate, which we calculated using QUAST (version 4.3) (81). Isolates were then classified into

TABLE 2 Description of statistical approaches[b]

Outcome variable	n	Analytical approach	Predictor variable	Random effect
Contingency table of ESC-R *E. coli* (presence/absence)	406	Fisher's exact test	species (dog/raccoon)	NA
ST richness	152	Bootstrapping	species	NA
Pairwise SNP distance of ESC-R *E. coli*	152	Univariable PERMANOVA	species	NA
ESC-R *E. coli* presence in raccoons (yes/no)	211	Multivariable binomial GLMM	private / public site, season (fall, winter, spring, summer), urban-suburban context (urban/suburban), urban context × private / public site	capture site, raccoon ID[a]
Pairwise SNP distance of ESC-R *E. coli*	152	Univariable PERMANOVA	host type (public park raccoon, private park raccoon, dog)	NA

[a]Variable was considered for inclusion as random effect in exploratory analyses but was found to contribute little to the overall variance ($p < 0.05$) and was thus excluded from analyses listed here.
[b]PERMANOVA, permutational multivariate analysis of variance; GLMM, generalized linear mixed model; ESC-R, extended-spectrum cephalosporin-resistant; ST, sequence type; SNP, single-nucleotide polymorphism; NA, not applicable.

different sequence types (STs) using mlst (https://github.com/tseemann/mlst) and the *in silico E. coli* PubMLST typing scheme. Associations between STs were visualized using minimum spanning trees, which were created in GrapeTree (82). To explore isolate similarity within STs, a core single-nucleotide polymorphism (SNP)-based phylogenetic analysis was performed. A detailed description can be found in reference 13. Briefly, trimmed reads were mapped to the *E. coli* K-12 laboratory strain MG1655 genome (accession number GCA_000005845.2), and recombinant regions were removed before generating a SNP distance matrix and constructing a maximum likelihood phylogenetic tree. The tree was visualized and annotated using the iTOL (Interactive Tree of Life) online software (83). Isolates that differed by less than 20 core SNPs were considered to be similar, as in references 71, 72, and 84–86.

The presence of ARG on assembled contigs was assessed using NCBI's BLASTn and the ResFinder database (88). An ARG was considered present if it had an identity of ≥90% and a coverage of ≥80%. For more information see reference 13.

Statistical analysis. (i) Objective 1: similarity of ESC-R *E. coli* isolated from raccoons and dogs based on prevalence, richness, and phylogenetic relatedness. The sample prevalence of ESC-R *E. coli* and 95% confidence intervals for raccoons and dogs were calculated using the "prevalence" package in R version 4.0.2 (87). Comparisons of the prevalence of ESC-R *E. coli* by species were performed using Fisher's exact test (Table 2). Using a similar approach to Mather et al. (89), the richness of ESC-R *E. coli* STs (number of unique STs found in raccoons and dogs) was compared between raccoon and dog populations by bootstrapping the raccoon sample ($n = 123$) to the size of the dog sample ($n = 29$) using 1,000 replicates. Deeper phylogenetic associations between ESC-R *E. coli* isolated from dogs and raccoons were explored by quantifying the pairwise SNP distance between isolates. Phylogenetic clustering by animal species (dog versus raccoon) was assessed by performing permutational multivariate analysis of variance (PERMANOVA) using the "adonis2" function in the "vegan" package (90) with the number of permutations set to 999. PERMANOVA can be used on any type of pairwise matrix (93) and can be used to identify factors shaping microbe phylogenetic associations (91). The assumption of homogeneity of variance was validated using the "betadisper" function in vegan.

(ii) Objective 2: difference in the probability of isolating ESC-R *E. coli* between raccoons sampled at public parks and raccoons sampled at private parks. The outcome variable for this analysis was presence of at least one ESC-R *E. coli* isolate in the feces of raccoons (yes or no) (Table 2). The interface of raccoons with dogs was quantified based on whether raccoons were sampled at private or public sites (private/public site). Associations were explored using a binomial generalized linear mixed model (GLMM) with a logit link function using the "lme4" package (92). Other predictors included season and urban-suburban context because previous work in this system found that both can influence the likelihood of isolating ESC-R *E. coli* from raccoons (13). We did not include raccoon age or sex as fixed effects because neither were expected to be important based on our previous work (13). The interaction between private/public site and urban-suburban context was also explored. Because 18 raccoons were captured more than once, we investigated the need for including "animal ID" as a random effect. To do this, we compared the Akaike information criterion (AIC) values between an intercept model with and without animal ID included as a random effect. There was no significant difference in AIC values between the two models (AIC = 319.49 and 317.9, $P = 0.52$), indicating that including animal ID as a random effect was not needed (Table 2). Capture site was included as a random effect to accommodate for any spatial autocorrelation in model residuals (Moran's I statistic post including capture site as a random effect: $z = -0.44$, $P = 0.67$).

(iii) Objective 3: phylogenetic similarity of ESC-R *E. coli* isolated from raccoons sampled at public parks, raccoons sampled at private parks, and dogs. The outcome variable in this analysis was pairwise SNP distance of ESC-R *E. coli*. The importance of the variable "host type" (i.e., public park raccoon, private park raccoon, or dog) at influencing the phylogenetic clustering of ESC-R *E. coli* was assessed by running a univariable PERMANOVA as in Objective 1 (Table 2).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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