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### Authors

Miller, Elizabeth A
Johnson, Timothy J
Omondi, George
et al.



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# Assessing Transmission of Antimicrobial-Resistant *Escherichia coli* in Wild Giraffe Contact Networks

**Elizabeth A. Miller,[a,b] Timothy J. Johnson,[b] George Omondi,[a] Edward R. Atwill,[c] Lynne A. Isbell,[d] Brenda McCowan,[c] Kimberly VanderWaal[a]**

[a]Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota, USA
[b]Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota, USA
[c]Department of Veterinary Health & Reproduction, School of Veterinary Medicine, University of California, Davis, California, USA
[d]Department of Anthropology and Animal Behavior Graduate Group, University of California, Davis, California, USA

**ABSTRACT** There is growing evidence that anthropogenic sources of antibiotics and antimicrobial-resistant bacteria can spill over into natural ecosystems, raising questions about the role wild animals play in the emergence, maintenance, and dispersal of antibiotic resistance genes. In particular, we lack an understanding of how resistance genes circulate within wild animal populations, including whether specific host characteristics, such as social associations, promote interhost transmission of these genes. In this study, we used social network analysis to explore the forces shaping population-level patterns of resistant *Escherichia coli* in wild giraffe (*Giraffa camelopardalis*) and assess the relative importance of social contact for the dissemination of resistant *E. coli* between giraffe. Of 195 giraffe sampled, only 5.1% harbored *E. coli* isolates resistant to one or more tested antibiotics. Whole-genome sequencing on a subset of resistant isolates revealed a number of acquired resistance genes with linkages to mobile genetic elements. However, we found no evidence that the spread of resistance genes among giraffe was facilitated by interhost associations. Giraffe with lower social degree were more likely to harbor resistant *E. coli*, but this relationship was likely driven by a correlation between an individual's social connectedness and age. Indeed, resistant *E. coli* was most frequently detected in socially isolated neonates, indicating that resistant *E. coli* may have a selective advantage in the gastrointestinal tracts of neonates compared to other age classes. Taken together, these results suggest that the maintenance of antimicrobial-resistant bacteria in wild populations may, in part, be determined by host traits and microbial competition dynamics within the host.

**IMPORTANCE** Antimicrobial resistance represents a significant threat to human health, food security, and the global economy. To fully understand the evolution and dissemination of resistance genes, a complete picture of antimicrobial resistance in all biological compartments, including natural ecosystems, is required. The environment and wild animals may act as reservoirs for anthropogenically derived resistance genes that could be transferrable to clinically relevant bacteria of humans and domestic animals. Our study investigated the possible transmission mechanisms for antimicrobial-resistant bacteria within a wild animal population and, more broadly, contributes to our understanding of how resistance genes are spread and maintained in natural ecosystems.

**KEYWORDS** *Escherichia coli*, antibiotic resistance, social network analysis, transmission dynamics, wildlife

Antimicrobial resistance (AMR) is a major global threat to public health and animal agriculture (1, 2). Ongoing misuse and overprescription of antibiotics creates selective pressure for AMR-encoding genes (ARGs) that impact the prevention and treatment of

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Address correspondence to Elizabeth A. Miller, millere@umn.edu.

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infections in both humans and domestic animals. Of further concern is the growing evidence that residual antibiotics and resistant bacteria may spread from clinical and agricultural settings into natural ecosystems, creating reservoirs of ARGs that could undergo horizontal gene transfer into novel opportunistic pathogens (3–6). Indeed, clinically relevant antibiotic-resistant bacteria have been identified in a wide range of natural habitats and wild animal species, including those at the human-agriculture-environment interface (for examples, see references 5 and 7 to 10). However, there are still large gaps in our understanding of the potential role natural ecosystems play in the emergence, maintenance, and dispersal of ARGs. We also know relatively little about what forces shape observed patterns of AMR in wild animal populations (3, 11–13), yet this information is critical for determining if intervention is required and subsequently how control strategies could be implemented (12).

Understanding ARG dissemination in wild animals requires knowledge, in part, of commensal bacterial transmission. Commensal bacteria in the host gastrointestinal (GI) tract can acquire and transfer ARGs with relative ease, and resistant bacterial clones are often used as indicators of AMR prevalence in GI bacterial populations (14–16). Recent work with humans and other animals suggests that these GI bacteria can spread between hosts via interactions, such as physical contact or shared environmental resources (17, 18). Thus, individuals with direct or indirect associations may share specific bacterial strains (19–21) or have more similar microbial communities (22–24).

By extension, physical contact or common environments may similarly promote the spread of antibiotic-resistant bacteria among human and animal hosts. Indeed, there is growing evidence that shared use of environmental resources may facilitate the transmission of resistant bacteria between domestic animals, human populations, environmental reservoirs, and wildlife (25–27). Within populations, however, there are still unanswered questions regarding the relative importance of AMR "spillover" events from humans or domestic animals compared to independent circulation of resistant bacteria within wild animals. Specifically, are repeated spillovers required to sustain AMR in a wild animal population or, after the initial introduction of an ARG from an anthropogenic source, does interhost transmission within the wild population contribute to AMR maintenance and dispersal? Research on within-species ARG transmission is still rare and primarily limited to studies with livestock (28, 29) and humans (30, 31). Comparable studies in wildlife systems would be of significant value for understanding the transmission dynamics of ARGs within natural ecosystems and in the absence of antibiotic use.

In this study, we used wild giraffe (*Giraffa camelopardalis*) to investigate the drivers shaping population-level patterns of antibiotic-resistant *Escherichia coli*. Giraffe sociality is characterized by fission-fusion social organization, where social group membership changes daily or even hourly as animals move through their environment (32–35). Variation in patterns of direct or indirect association could influence how antibiotic-resistant bacteria are spread through the population. Commensal *E. coli* is commonly found in the GI tracts of a wide range of animals and is disseminated via host contact with fecally contaminated environmental resources or direct host-to-host contact (36–39). Further, both commensal and pathogenic *E. coli* organisms are thought to be particularly important contributors to the global spread of antimicrobial resistance genes via mobile genetic elements (MGEs) (40, 41). Prior research in the same giraffe population found that social contact networks predicted *E. coli* sharing between individuals; giraffe that were more strongly connected in the co-occurrence network were more likely to harbor the same *E. coli* strains than giraffe rarely observed together (21). As a follow-up to this work, we investigated whether giraffe contact networks similarly predict patterns of antibiotic-resistant *E. coli* among individuals. First, we identified which host characteristics, including metrics of both host social and spatial connectivity, influence the likelihood of a giraffe harboring resistant *E. coli*. We then tested whether giraffe contact networks could promote the dissemination of ARGs between individuals. If social or spatial contact networks predict patterns of observed ARGs within the giraffe population, this would suggest that social associations may

**TABLE 1** Antibiotic resistance phenotype of *E. coli* isolates from giraffe

| Isolate[a] | Antibiotic resistance phenotype[b] |
|---|---|
| $G89_3$ | AMX, OXY, SXT |
| $G89_4$ | AMX, OXY, SXT |
| $G363_1$ | OXY |
| $G363_2$ | OXY |
| $G366_2$ | AMX |
| $G670_1$ | CTX,[c] AMX |
| $G670_3$ | CTX,[c] AMX |
| $G670_4$ | CTX,[c] AMX |
| $G674_1$ | OXY |
| $G674_3$ | OXY |
| $G678_2$ | AMX |
| $G678_4$ | AMX |
| $G684_4$ | OXY, SXT[c] |
| $G685_3$ | AMX, OXY, SXT |
| $G685_4$ | AMX, OXY, SXT |
| $G688_1$ | OXY |
| $G688_2$ | OXY |
| $G688_3$ | OXY |
| $G688_4$ | OXY |
| $G689_1$ | AMX, OXY, SXT |
| $G689_2$ | AMX, OXY, SXT |

[a]The isolates are labeled by giraffe identification number with the isolation number in the subscript.
[b]Abbreviations: AMX, amoxicillin; CTX, cefotaxime; OXY, oxytetracycline; SXT, co-trimoxazole.
[c]The MIC was below the clinical breakpoint defined by EUCAST but above the ECOFF.

facilitate the circulation of resistant bacteria within wildlife populations. Taken together, these results advance our understanding of ARG transmission dynamics, including the forces responsible for the distribution of AMR in natural ecosystems.

## RESULTS

**Antimicrobial resistance was rare in giraffe *Escherichia coli* and unrelated to subtype.** We conducted antibiotic susceptibility testing on 765 *E. coli* isolates from 195 giraffe inhabiting the Ol Pejeta Conservancy (OPC) wildlife reserve in Kenya. Of these isolates, antibiotic resistance was detected in only 2.7% of isolates ($n = 21$ isolates) from 5.1% of giraffe ($n = 10$ individuals) (Table 1). Multidrug-resistant isolates (resistant to three or more antibiotic classes [42]) were found in 30% of AMR-positive giraffe ($n = 3$), with the most common phenotype consisting of resistance to amoxicillin, oxytetracycline, and co-trimoxazole (Table 1). Three isolates from a single giraffe (G670) exhibited cefotaxime MICs above the epidemiological cutoff value (ECOFF) but not the clinical breakpoint, suggesting resistance to cefotaxime may be emerging in wild-type *E. coli* but is not yet clinically relevant. Similarly, isolate $G684_4$ exhibited a co-trimoxazole MIC above the ECOFF but not the clinical breakpoint. None of the 765 *E. coli* isolates exhibited resistance to florfenicol or gentamicin.

Within an individual giraffe, resistant isolates exhibited the same AMR profiles and were typically the same *E. coli* genomic subtype (mean pairwise genetic similarity based on BOX-PCR: 97.8% $\pm$ 1.6%). This suggests that multiple clones of a single *E. coli* strain were likely collected and screened from individual giraffe. However, between giraffe, genetic similarity was not an indication of matching AMR profiles (mean genetic similarity: 55.6% $\pm$ 30.5%).

**Acquired resistance genes were common and found in proximity to mobile genetic elements.** Given that resistant isolates within an individual giraffe were the same *E. coli* genomic subtype, a single representative resistant isolate was selected from each AMR-positive giraffe for whole-genome sequencing and *de novo* assembly ($n = 10$ isolates). The average read depth per isolate was 28$\times$, with a mean of 248 scaffolds of $\geq$500 bp after assembly (see Table S1 in the supplemental material). *In silico* phylotyping revealed that resistant isolates belonged to all four main phylogroups and accessory group E (Table 2). Phylotypes B1 and D accounted for 40% ($n = 4$) and 30% ($n = 3$) of the isolates, respectively, while phylotypes A, B2, and E were each repre-

**TABLE 2** Genomic characteristics of *E. coli* isolates displaying phenotypic resistance to at least one antibiotic

| Isolate[a] | Sequence type | Phylotype | Scaffold identifier | Size (bp) | Acquired resistance gene(s) (GenBank accession no.) | Genetic context[b] | Scaffold group[c] |
|---|---|---|---|---|---|---|---|
| $G89_4$ | ST1670 | D | NODE_130 | 8,626 | *catA1* (V00622), *dfrA7* (JF806498), *sul1* (various) | *intI1*, *qacEΔ1*, Tn*3* transposase, *tnpM* | 1 |
| | | | NODE_156 | 4,584 | *strA* (AF321551), *strB* (M28829), *sul2* (HQ840942) | IncQ1 plasmid | 2 |
| | | | NODE_162 | 4,066 | *tet*(A) (AJ517790) | Unknown | 3 |
| | | | NODE_193 | 2,029 | $bla_{TEM-1}$ (JF910132) | *tnpR* | 4 |
| $G363_1$ | ST5281 | E | NODE_115 | 6,198 | *strA* (M96392), *strB* (M28829), *sul2* (AY034138) | Unknown | 5 |
| | | | NODE_125 | 4,928 | *tet*(B) (AP000342) | Clustered with Tn*10*; *tetR*, *tet*(C), *tet*(D) | 6 |
| $G366_2$ | ST1670 | D | NODE_24 | 65,775 | *catA1* (V00622), *dfrA7* (JF806498), *sul1* (various) | *intI1*, *qacEΔ1*, *insA*, IS1 *orfA*, IS*1* transposase, *intS* | 1 |
| | | | NODE_140 | 4,640 | *strA* (AF321551), *strB* (M28829), *sul2* (HQ840942) | IncQ1 plasmid | 2 |
| | | | NODE_48 | 36,946 | *tet*(A) (AJ517790) | *tnsB* | 3 |
| | | | NODE_166 | 2,085 | $bla_{TEM-1}$ (JF910132) | *tnpR* | 4 |
| $G670_1$ | ST5419 | B1 | | | | | |
| $G674_1$ | ST95 | B2 | NODE_82 | 5,615 | *tet*(A) (AJ517790) | Unknown | |
| $G678_2$ | ST1433 | A | NODE_31 | 63,379 | $bla_{TEM-1}$ (JF910132) | IncK plasmid, *tnpA*, *tnpR* | 4 |
| $G684_4$ | ST947 | B1 | NODE_123 | 6,217 | *strA* (M96392), *strB* (M28829), *sul2* (AY034138) | Unknown | 5 |
| | | | NODE_132 | 4,910 | *tet*(B) (AP000342) | Clustered with Tn*10*; *tetR*, *tet*(C), *tet*(D) | 6 |
| $G685_3$ | ST337 | B1 | NODE_73 | 14,011 | *dfrA5* (X12868), *tet*(A) (AJ517790) | Plasmid (unclassified), *intI1*, Tn*3* transposase, *tnpM* | |
| | | | NODE_112 | 2,952 | *strA* (AF321551), *strB* (M28829), *sul2* (HQ840942) | Unknown | 2 |
| | | | NODE_114 | 2,017 | $bla_{TEM-1}$ (JF910132) | Plasmid (unclassified), *tnpR* | 4 |
| $G688_1$ | ST937 | B1 | NODE_85 | 5,632 | *strA* (M96392), *strB* (M28829), *sul2* (AY034138) | Unknown | 5 |
| | | | NODE_90 | 4,902 | *tet*(B) (AP000342) | Clustered with Tn*10*; *tetR*, *tet*(C), *tet*(D) | 6 |
| $G689_1$ | ST1670 | D | NODE_119 | 8,682 | *catA1* (V00622), *dfrA7* (JF806498), *sul1* (various) | *intI1*, *qacEΔ1*, Tn*3* transposase, *tnpM* | 1 |
| | | | NODE_147 | 4,640 | *strA* (AF321551), *strB* (M28829), *sul2* (HQ840942) | IncQ1 plasmid | 2 |
| | | | NODE_40 | 43,030 | *tet*(A) (AJ517790) | *tnsB* | 3 |
| | | | NODE_181 | 2,085 | $bla_{TEM-1}$ (JF910132) | *tnpR* | 4 |

[a]The isolates are labeled by giraffe identification number, with the isolation order number as the subscript.
[b]Lists any genes identified via the INTEGRALL (115), ISfinder (116), PlasmidFinder (117), and NCBI nucleotide (https://www.ncbi.nlm.nih.gov/nucleotide/) databases associated with mobile genetic elements, including integrons, transposons, insertion sequences, plasmids, and phages.
[c]Scaffold groups were determined from BLAST alignments of ≥99% similarity across ≥90% of the shorter scaffold sequence.

sented by one isolate (Table 2). *In silico* multilocus sequence type (MLST) analysis identified seven *E. coli* sequence types (STs), including ST1670, which accounted for all three of the phylotype D isolates (Table 2).

A total of 10 different acquired ARGs were identified, with an average of 4 ARGs per isolate (range: 0 to 8 ARGs [Table 2]). Genes conferring resistance to sulfonamides had the highest prevalence (*sul1* and *sul2*; $n = 9$ isolates), followed by genes conferring resistance to tetracyclines [*tet*(A) and *tet*(B); $n = 8$], aminoglycosides (*strA* and *strB*; $n = 7$), and beta-lactams ($bla_{TEM-1}$; $n = 5$). In general, ARGs identified in each isolate reflected the observed phenotypic AMR profiles (Tables 1 and 2). However, we did not identify any acquired ARGs in isolate $G670_1$ that could be responsible for observed resistance to amoxicillin or the elevated cefotaxime MIC. Additionally, isolate $G366_2$ carried genes that typically confer resistance to oxytetracycline [*tet*(A)] and co-trimoxazole (*sul1*, *sul2*, and *dfrA7*) but did not exhibit phenotypic resistance to either antibiotic. Finally, although the presence of *sul2* in isolate $G684_4$ could account for a co-trimoxazole MIC above the ECOFF, the same gene was also identified in two other isolates that did not exhibit elevated co-trimoxazole MICs ($G688_1$ and $G363_1$) (Tables 1 and 2).

Sequence alignments of ARG-carrying scaffolds identified six scaffold groups where pairwise scaffold similarity was ≥99% for at least 90% of the shorter scaffold's length (Table 2). Two of these scaffold groups were characterized by the presence of a *sul2-strAB* gene cluster (scaffold groups 2 and 5), while scaffold group 1 carried the genes *catA1*, *dfrA7*, and *sul1* (Table 2). The remaining three scaffold groups (3, 4, and 6) carried one ARG each: *tet*(A), *tet*(B), and $bla_{TEM-1}$, respectively (Table 2). Genes associated with mobile genetic elements (MGEs), such as those encoding transposases, resolvases, integrases, and plasmid replicons, were found in 71% of all ARG-carrying scaffolds ($n = 17$) across 50% of isolates ($n = 5$) (Table 2). Of particular note was the presence of the class 1 integron-integrase gene, *intI1*, on all members of scaffold group 1, in addition to a unique scaffold carrying ARGs *tet*(A) and *dfrA5* (Table 2). Interestingly, the group 1 scaffold from isolate $G366_2$ also carried the phage-related integrase gene, *intS*, suggesting the presence of a phage. Six of the 17 ARG-carrying scaffolds were identified as plasmids across five isolates, including the IncQ1 plasmid in three *sul2-*

**TABLE 3** Univariate model results for potential social and ecological predictors of *E. coli* antimicrobial resistance in giraffe

| Covariate | Coefficient | *P* value | ΔAICc | AICc weight |
|---|---|---|---|---|
| Social degree | −0.19 | <0.001 | 0.0 | 0.93 |
| Age | | | 6.0 | 0.05 |
| Juvenile | −3.30 | 0.001 | | |
| Subadult | −4.34 | <0.001 | | |
| Adult | −4.28 | <0.001 | | |
| Home range size | −0.03 | 0.003 | 8.6 | 0.01 |
| Spatial degree | −0.06 | 0.004 | 10.1 | <0.01 |
| Sex: male | 0.03 | 0.59 | 12.7 | <0.01 |
| Social betweenness | −0.001 | 0.13 | 13.1 | <0.01 |
| Spatial betweenness | −0.001 | 0.18 | 13.7 | <0.01 |
| Intercept only | −2.87 | <0.001 | 23.5 | <0.01 |
| River side: west | −0.14 | 0.31 | 24.7 | <0.01 |

*strAB* isolates and the IncK plasmid in one $bla_{TEM-1}$ isolate (Table 2). All members of the $bla_{TEM-1}$-positive scaffold group also carried the gene *tnpR*, which encodes a transposon gamma-delta resolvase typically found in Tn*3* family transposons.

**Neonates showed higher antimicrobial resistance than other age classes.** We investigated the importance of host-level social and ecological factors for predicting antimicrobial-resistant *E. coli* in giraffe using univariate and multivariate Firth bias-reduced logistic regressions (Tables 3 and 4). In both univariate and multivariate models, social degree (i.e., number of social contacts) was the single most important predictor of AMR risk, accounting for more than 90% of Akaike's information criterion corrected for small sample size (AICc) weight in univariate models and included in all three best-fit multivariate models (Tables 3 and 4). Specifically, giraffe with lower social degree were significantly more likely to carry at least one resistant *E. coli* isolate (*P* value $\leq$ 0.001). Age class was the second most important predictor in univariate models, with 5% of AICc weight (Table 3). Neonates were significantly more likely to carry at least one resistant *E. coli* isolate than juveniles, subadults, and adults (Tables 3 and 5). However, in multivariate models, only one of the three best-fit models contained age (Table 4). This result was somewhat surprising given that 71% of all neonates (5/7 giraffe) carried at least one resistant isolate and neonates accounted for 50% of all AMR-positive giraffe (Table 5). One possible explanation for the minimal importance of age in regression models is that other factors, such as social degree, may have reflected additional information about age variation not captured by age classes. For example, among giraffe with known ages (i.e., neonates and juveniles), social degree was highly correlated with age (Pearson's $r = 0.80$; Pearson's $r = 0.93$ if restricted to <150 days of age) (Fig. 1), and younger individuals were more likely to carry resistant *E. coli* (Wilcoxon rank sum test: $W = 3$; *P* value $< 0.0001$).

AMR risk was also related to small home range size and low spatial degree in univariate models (Table 3), but neither factor was consistently present or significant in the best-fit multivariate models (Table 4). Although social and spatial betweenness accounted for less than 1% of AICc weight in univariate model comparisons (Table 3),

**TABLE 4** Coefficients of the best-fit models for antibiotic-resistant *E. coli* risk in giraffe[a]

| | | | Covariate | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Age | | | |
| Model | ΔAICc | AICc weight | Social degree | Social betweenness | Spatial betweenness | Home range size | Juvenile | Subadult | Adult | Spatial degree |
| A | 0 | 0.50 | −0.20[d] | 0.004[b] | −0.0005 | −0.02[b] | | | | |
| B | 0.87 | 0.32 | −0.17[c] | 0.006[c] | −0.008[b] | 0.01 | −1.82 | −3.72[b] | −3.62[c] | |
| C | 2.33 | 0.16 | −0.21[d] | 0.005[b] | −0.004 | | | | | −0.03 |

[a]Only models receiving >5% AICc weight are shown. AICc weights were calculated relative to all univariate and multivariate models.
[b]$P \leq 0.05$.
[c]$P \leq 0.01$.
[d]$P \leq 0.001$.

**TABLE 5** Prevalence of antibiotic-resistant *E. coli* in giraffe fecal samples by age class

| Antibiotic | No. of resistant samples (%)[a] | | | |
|---|---|---|---|---|
| | Neonate ($n = 7$) | Juvenile ($n = 19$) | Subadult ($n = 17$) | Adult ($n = 152$) |
| Any | 5 (71.4) | 1 (5.3) | 0 | 4 (2.6) |
| Amoxicillin | 3 (42.9) | 0 | 0 | 3 (2.0) |
| Cefotaxime | 0 | 0 | 0 | 0 |
| Florfenicol | 0 | 0 | 0 | 0 |
| Gentamicin | 0 | 0 | 0 | 0 |
| Oxytetracycline | 4 (57.1) | 1 (5.3) | 0 | 2 (1.3) |
| Co-trimoxazole | 2 (28.6) | 0 | 0 | 1 (0.7) |

[a]For each antibiotic, the first number represents the number of giraffe with ≥1 resistant *E. coli* isolate and the related percentage for the given age class is indicated in parentheses.

both factors were included in all three best-fit multivariate models (Table 4). Specifically, giraffe AMR risk increased with increasing social betweenness and decreasing spatial betweenness (Table 4). However, the values of betweenness regression coefficients were small (Table 4), suggesting that the effect of social and spatial betweenness on AMR risk was at most minor compared to other factors.

**Contact networks were unrelated to patterns of antimicrobial resistance.** We next assessed the relevance of giraffe contact networks for the transmission of ARGs between giraffe. Plots of both social and spatial networks suggest there was relatively little clustering of AMR-positive giraffe (Fig. 2). Network *k*-tests and path tests (43) were conducted on social and spatial networks for each scaffold group identified in at least two AMR-positive giraffe. All tests failed to reject the null hypothesis that ARG-positive giraffe were distributed randomly in the networks (Bonferroni adjusted *P* values $>$ 0.99).

## DISCUSSION

To date, considerable research has focused on ARG dissemination between natural ecosystems and humans or domestic animals (for examples, see references 27 and 44 to 46). However, less attention has been paid to understanding whether ARGs circulate independently within wild animal populations after an introductory anthropogenic spillover event (but see references 27 and 47). In this study, we investigated the potential drivers shaping patterns of antimicrobial-resistant *E. coli* within a population of wild giraffe. While we did find clinically resistant *E. coli* in this population, social network analyses provided little evidence for the spread of acquired ARGs via interhost associations. Instead, the presence of AMR in this population may be primarily driven

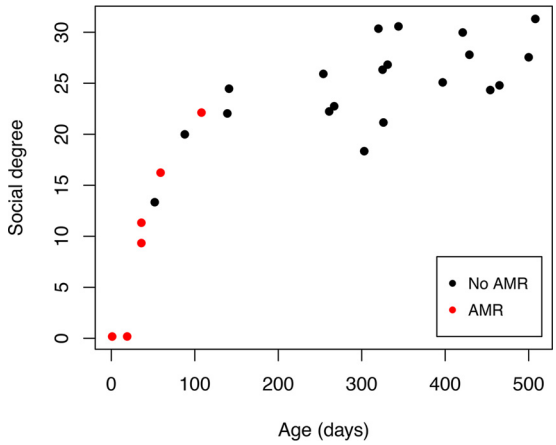


**FIG 1** Relationship between social degree and age in days. Each point represents one giraffe. Red points indicate giraffe harboring at least one antimicrobial-resistant *E. coli* isolate.

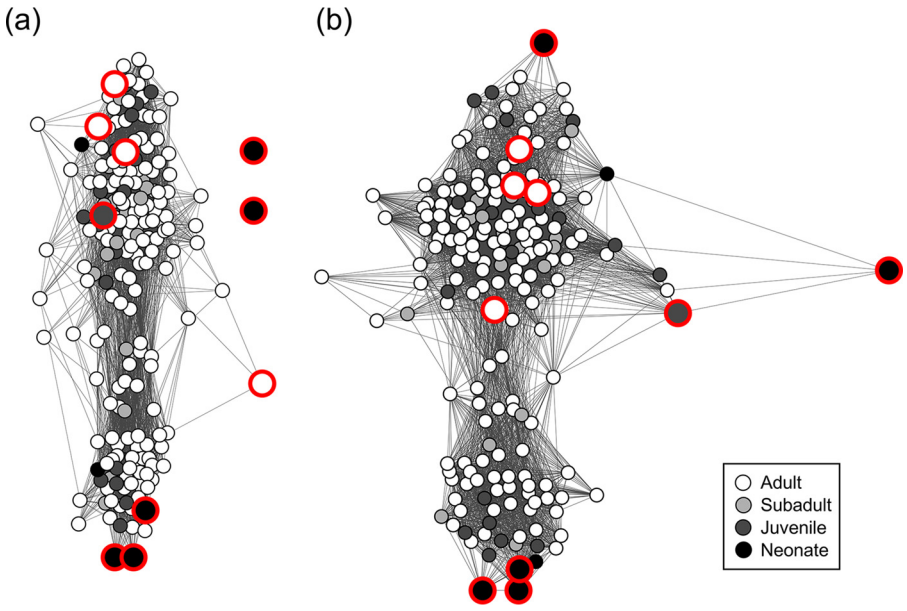


**FIG 2** Networks of giraffe association strength (a) and home range overlap (b). Enlarged nodes outlined in red indicate giraffe yielding at least one antimicrobial-resistant *E. coli* isolate.

by repeated spillover of resistant bacteria from anthropogenic sources or related to other potential selection pressures for ARGs.

Giraffe home ranges overlap extensively with herds of domestic cattle, and all animals within OPC share common water sources that may act as environmental reservoirs of ARGs and hotspots of transmission from cattle to wild animals. While our current results alone cannot establish whether the resistant *E. coli* organisms were directly transmitted from the local cattle population, a number of our findings do provide more general evidence that AMR in this giraffe population was primarily derived from anthropogenic sources. First, 9 of the 10 *E. coli* isolates we sequenced carried at least one acquired ARG, and all of these ARGs have been previously identified in *E. coli* from both humans and domestic cattle in East Africa (48–52). Further, prior research on AMR at human-livestock-wildlife interfaces suggests that the presence of these ARGs in wild animals is closely associated with anthropogenic land usage (27, 47, 53–55). Second, although the dominant B1 phylogroup of our sequenced isolates is typical for *E. coli* in both domestic and wild herbivorous animals (36, 56), two other isolates belonged to phylogroups A and B2, the dominant phylogroups of human populations (36, 57). Both isolates also had MLST sequence types (ST1433 and ST95) previously isolated from humans and frequently associated with extraintestinal diseases, including urinary tract and bloodstream infections (58, 59). Third, we found that four of our sequenced isolates carried the class 1 integron-integrase gene, *intI1*. This MGE-associated gene plays a major role in the global spread of ARGs and is thought to be a reliable genetic marker of anthropogenic pollution (60, 61). Taken together, these results suggest that giraffe AMR is, in large part, derived from anthropogenic sources. However, the specific local sources of AMR—whether cattle or another unknown source—are yet to be determined.

The genetic characterization of our resistant isolates also provides evidence for both the horizontal transfer of ARGs between bacterial strains and the clonal spread of ARG-carrying *E. coli*. On the one hand, we observed multiple instances where isolates with distinct genetic backgrounds harbored the same ARGs in similar genetic contexts. For example, scaffold group 5, which carried the *sul2-strAB* gene cluster, was found in three isolates with three different MLST sequence types. While we could not identify any MGEs associated with this scaffold group, prior studies found that the *sul2-strAB* gene cluster is part of a cassette and typically found on plasmids in a wide range of

Gram-negative bacteria, including *E. coli* (62, 63). On the other hand, the ST1670 genomic subtype found in three different giraffe shared matching ARGs that were carried by the same four scaffold groups, which suggests that they may have descended from the same recent ancestor. This is in contrast to prior research on a sympatric population of cattle and wild buffalo in southern Africa that found the spread of ARGs was primarily independent of *E. coli* strain sharing between individuals (27). Together, our findings regarding both horizontal gene transfer and clonal transmission of ARGs suggest that both mechanisms play a role in the maintenance of AMR in this wild animal population and highlight the potential complexity of ARG spread.

The overall low prevalence of AMR identified in this population suggests that spillover from local humans and livestock may be a relatively rare occurrence or that there are simply low reservoirs of resistance to be shared between populations. This is somewhat surprising given reports of high antimicrobial use and AMR in both humans and livestock in Kenya (64–68) and high prevalence of resistant bacteria observed in previous studies of East African wildlife (8, 9, 69). In part, this variability in AMR prevalence between studies may be explained by differences in dietary niche between host species. Specifically, the type and location of food consumed may affect the level of exposure to residual antibiotics and resistant bacteria. For example, carnivores typically exhibit the highest AMR risk, possibly due to trophic accumulation of ARGs through the food chain, while herbivores have the lowest risk (3, 8, 70). Giraffe exposure to AMR may be particularly low because their diet primarily consists of leaves from trees and shrubs, areas where antibiotic contamination and resistant bacteria associated with the host GI tract are less likely to exist.

Alternatively, the low AMR prevalence in this study compared to findings for other African wildlife may simply be an unintended consequence of our sampling design. The GI tract typically harbors a single dominant strain of *E. coli* that constitutes more than half of the total isolated colonies (71, 72). Since many AMR mutations or plasmids can reduce fitness in the absence of antibiotic selection pressures, resistant strains may be outcompeted by nonresistant strains with higher fitness (73, 74). Thus, finding dominant strains with AMR phenotypes may be relatively rare in wild animals where selection for AMR is low (27). Genotyping of *E. coli* used in this study identified, on average, only 1.7 unique subtypes per giraffe (21). Consequently, the majority of susceptibility testing was probably conducted on the dominant *E. coli* strains, where resistance may have been present but rare. A sampling design that includes susceptibility testing of both dominant and subdominant isolates might have revealed higher levels of AMR within the giraffe population. For example, work with wild African buffalo found AMR in only one dominant *E. coli* strain but identified resistance in many subdominant isolates (27). Similar findings have also been reported for both livestock (75) and humans (76, 77). Assessment of these rare, subdominant strains could be more effectively achieved either by initially culturing fecal samples in the presence of antibiotics or through metagenomic sequencing approaches.

AMR prevalence may also be low if the antibiotics we selected were not representative of AMR selection pressures in humans, livestock, and wildlife within the study area. However, it is worth noting that the two antibiotics most frequently used to treat cattle and wildlife (amoxicillin and oxytetracycline; G. Omondi, unpublished data) were also the two most common AMR phenotypes we identified in the giraffe population. Other antibiotics with higher rates of improper usage, such as those obtained over the counter by nonprofessionals near OPC, would be worth including in susceptibility testing for future surveys of AMR in this area (68).

Despite the relatively low prevalence of giraffe AMR, regression model results indicated that there was a compelling relationship between AMR risk and individual-level measures of social connectedness. In particular, individuals with fewer social links to other giraffe were more likely to harbor resistant *E. coli*. However, it is unclear whether this relationship reflects a real effect of social connectivity on AMR or whether it captures variation in another host characteristic not accounted for in our data set. The latter explanation seems more likely given that socially mediated transmission should

result in giraffe with higher social degree experiencing higher AMR risk. Indeed, at the network level, neither social nor spatial contact networks explained the observed pattern of AMR-positive giraffe. Instead, we observed strong age-related patterns of AMR, with the highest AMR risk occurring in giraffe younger than 3 months. During the first few months of life, and particularly during the first 2 weeks, calves are kept relatively isolated from other giraffe and consequently have few social associations and small home ranges (78, 79). Thus, our observed relationship between social and spatial connectedness and AMR risk may simply reflect these early months when associations are less frequent and AMR is high. Future work disentangling age-related social and ranging behaviors from other aspects of social and spatial association will provide a more complete picture of the host traits that predispose certain groups to acquire, maintain, and spread ARGs.

The high likelihood of identifying resistant *E. coli* in neonate giraffe compared to other age classes suggests that age may be an important predictor of AMR risk in giraffe. In young giraffe, exposure to residual antibiotics is presumably low, particularly while neonates are exclusively nursing. Therefore, the high prevalence of AMR in neonates compared to other age classes is unlikely to be caused by differential selection pressures related to antibiotic exposure. Instead, our results suggest that resistant *E. coli* strains may have a selective advantage over susceptible strains within the neonate GI tract. Some ARGs may be genetically linked to advantageous genes that enhance the fitness of resistant strains, such as encoding mechanisms of colonization, adhesion, or reproduction (80, 81). For example, the milk diet of giraffe neonates could create a niche for bacterial strains that carry genes for enhanced acquisition or utilization of milk-associated nutrients and antimicrobial resistance (82, 83). As neonate feeding behavior shifts from nursing to extensive browsing between 2 and 4 months of age (78, 79), the selective advantage of these strains would be lost and the abundance of resistant bacteria would subsequently decrease (84). Indeed, our observation that AMR prevalence was higher in neonates (0 to 3 months) than in other giraffe age classes provides support for this hypothesis, but further culture and genome-based work would be required to identify the underlying mechanism(s) responsible for the apparent age-related differences in AMR. It is also worth noting that similar age-related distributions of AMR have also been observed in both humans and domestic animals (for examples, see references 85 to 88)—even in the absence of antibiotic selection pressures (82)—but the mechanisms underlying these patterns remain largely unknown.

Overall, our results indicate that the presence of AMR in this wild giraffe population is primarily due to dissemination of resistant *E. coli* strains and/or ARGs from local anthropogenic sources and not independent circulation of ARGs among giraffe. However, our finding that AMR risk differs between giraffe age classes suggests that ARG spread is not a completely random process and that the persistence of resistant strains may be dependent on host traits and competition dynamics of the gut microbial community. To date, the potential hazards of wild animal reservoirs of acquired ARGs have yet to be determined. Yet the mere presence of clinically resistant *E. coli* in natural ecosystems warrants concern and investigation into the potential consequences for both humans and domestic animals. Future AMR research should therefore include studies of wildlife and environmental reservoirs, with a focus on the role that natural ecosystems may play in the emergence, maintenance, and global spread of AMR.

## MATERIALS AND METHODS

**Study population and field observations.** This study was conducted at Ol Pejeta Conservancy (OPC), a wildlife reserve located in Laikipia, Kenya (0°N, 36°56′E) that integrates commercial cattle ranching with wildlife conservation. The reserve is bisected by the Ewaso Ng'iro river, with the western side home to wide-ranging OPC cattle herds and the eastern side featuring small clusters of cattle and proximity to local villages and their livestock. All giraffe within OPC at the time of the study ($n = 212$) were individually recognized based on unique spot patterns on their necks. Age class (neonate, <3 months; juvenile, 3 months to 1.5 years; subadult, 1.5 to 4 years; and adult, >4 years) for each giraffe was established according to physical attributes and age-associated behaviors (21, 78, 79). Approximate birth dates were known for all neonates and juveniles. The population exhibited a 50:50 sex ratio,

although the sex of two neonates born at the end of the observation period was not determined. At the end of the study period, the giraffe population of OPC consisted of 160 adults, 20 subadults, 21 juveniles, and 11 neonates.

Behavioral observations were carried out in OPC from 21 January to 2 August 2011 as described elsewhere (21). Briefly, giraffe were located by driving daily routes through different regions of OPC. Giraffe social group membership was determined by proximity to other group members and/or movement of individuals in a common direction (35, 89, 90). In total, there were 1,089 sightings of giraffe groups during the study period, with each giraffe observed an average of 31.1 ± 7.6 (mean ± standard deviation [SD]) times (approximately once per week).

This research was approved by Kenya's National Council for Science and Technology (permit NCST/RRI/12/1/MAS/147) and the UC Davis Institutional Animal Care and Use Committee (protocol no. 15887).

**Contact network construction.** We constructed two giraffe contact networks based on (i) social associations and (ii) spatial overlap of home ranges, with the same set of individuals included in both networks ($n = 193$). For the social network, patterns of association were established from group membership, with pairs of giraffe linked if they were observed in the same social group at least once. Network connections were weighted according to association strength (AS), which was calculated as the number of sightings where a giraffe pair was observed in the same group divided by the total number of times they were seen together or apart (21). Because giraffe mothers tend to isolate their calves from other giraffe for the first 1 to 3 weeks postpartum (78, 79), two neonates born at the end of field observations were linked only to their mothers.

The spatial network was constructed based on the extent of home range overlap between individuals (21). Network connections were weighted according to the proportion of overlap. Home range boundaries of each giraffe were mapped using a fixed-kernel utilization distribution of Global Positioning System (GPS) coordinates recorded during sightings. Each giraffe's core home range was calculated using a 75% kernel density estimation (91). Average home range size ranged from 16.9 ± 13.4 km$^2$ for neonates to 95.7 ± 3.3 km$^2$ for adult males.

For both social and spatial networks, we calculated two standard measures of network connectivity for each individual: weighted degree and weighted betweenness (92). Degree is defined as the number of individuals to which the focal individual is connected (93). Weighted degree (here social/spatial degree) is the extension of degree for weighted networks and accounts for both the number of linked individuals and the weight of those links (92). Betweenness measures the extent a focal individual falls on the shortest paths between other pairs of individuals in the network (93). For weighted networks, the shortest paths used to calculate betweenness are based on the sum of connection weights (92). Previous work has shown that both metrics positively correlate with microbial diversity and bacterial subtype sharing (21, 22, 94), as well as pathogen infection risk (95, 96), suggesting that individuals with high degree or betweenness may have elevated opportunities for exposure to antibiotic-resistant bacteria compared to those with low network connectivity.

**Fecal sample collection and DNA fingerprinting.** Detailed methods relating to fecal sample collection, *E. coli* isolation, and genetic analysis are described elsewhere (21). Briefly, fecal samples from 195 giraffe were collected between 10 August and 11 September 2011 (after the completion of behavioral observations). Collected samples were streaked onto *E. coli* selective CHROMagar EC agar (CHROMagar, Paris, France) and incubated overnight at 37°C. Up to six *E. coli* colonies (range, 2 to 6; median, 4) were randomly selected from each incubated sample, subcultured, and frozen for transport to the United States. To determine the genetic profile of each isolate, densitometric curves were generated using the banding patterns from BOX-PCR and gel electrophoresis (97–99). Genetic similarity of each isolate to all others was subsequently calculated through pairwise comparisons of densitometric curves using the Pearson product-moment correlation coefficient. Two isolates were interpreted as the same *E. coli* subtype if they were at least 90% similar (21).

**Antimicrobial susceptibility testing.** All *E. coli* isolates ($n = 765$) were tested for sensitivity to six antibiotics: amoxicillin (beta-lactam: penicillin), cefotaxime (beta-lactam: third-generation cephalosporin), florfenicol (phenicol), gentamicin (aminoglycoside), oxytetracycline (tetracycline), and cotrimoxazole (sulfonamide/dihydrofolate reductase [DHFR] inhibitor). These antibiotics were selected based on reports of antibiotic use in Kenya and recent research in eastern and southern Africa demonstrating acquired resistance in *E. coli* from local livestock and wild animals (8, 27, 48, 49, 65, 68). In addition, amoxicillin and oxytetracycline were the primary antimicrobial treatments used by OPC in cattle and wild animals (G. Omondi, unpublished data).

Sensitivity testing was performed using broth microdilution in a two-step process following methods established by the Clinical and Laboratory Standards Institute (CLSI) (100) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (101). First, isolates were tested against a single concentration of each antibiotic based on the epidemiological cutoff values (ECOFFs) determined for *Enterobacteriaceae* by EUCAST (102). ECOFFs represent the MICs that separate wild-type bacterial populations from strains with acquired resistance mechanisms (102). While these values do not provide information on the clinical relevance of resistance *per se*, they are useful for detection of AMR emergence in wild-type populations (102). Thus, an isolate was described as "tentatively resistant" if bacterial growth was observed in the presence of the antibiotic at the ECOFF concentration. In step two, all isolates tentatively classified as resistant from step one were tested for sensitivity across a range of 10 antibiotic concentrations using standard broth microdilution methods described by Wiegand et al. (103). Any isolate with a MIC above the established EUCAST clinical breakpoint was considered resistant (104). *E. coli* ATCC

25922 was used as a quality control strain in both steps of the sensitivity screen, as recommended by the CLSI and EUCAST (100, 101).

**Whole-genome sequencing and analyses.** DNA was extracted from one resistant isolate per giraffe using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). DNA libraries were prepared and multiplexed with the NexteraXT library preparation kit (Illumina, San Diego, CA) and subjected to paired-end whole-genome sequencing on the Illumina MiSeq platform with 300-bp read lengths. Raw sequences were trimmed and quality filtered using Trimmomatic v 0.33 (105), and sequence quality was assessed with FastQC v 0.11.7 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Average read depth and genome coverage were determined by mapping reads to the *E. coli* K-12 strain MG1655 chromosome (NCBI reference sequence NC_000913.3) using the Burrows-Wheeler Aligner v 0.7.17 (106) and SAMtools v 1.5 (107). *De novo* genome assemblies of high-quality reads were performed with SPAdes v 3.10.0 (108) following the assembly selection procedure of Ahlstrom et al. (47).

Prokka v 0.7.17 (109) was used to identify open reading frames (ORFs) on assembly scaffolds of ≥500 bp. *In silico* phylotyping of resistant isolates was based on the Clermont scheme (110) and performed using the web-based service ClermonTyper (http://clermontyping.iame-research.center/) (111). *In silico* MLST was used to identify isolate sequence types and performed using the Center of Genomic Epidemiology web-based MLST 2.0 method (112) (https://cge.cbs.dtu.dk/services/MLST/). Acquired resistance genes were identified by aligning ORFs to the ResFinder database (downloaded 22 April 2018) (113) using BLASTN (114) with an E value threshold of 1e−10, query identity of ≥99%, and query coverage of ≥85%. The similarity between ARG-carrying scaffolds from different isolates was assessed by pairwise BLASTN alignments. Scaffolds were assigned to the same scaffold cluster if their alignment exhibited ≥99% similarity across ≥90% of the length of the shorter scaffold sequence. To determine the genetic context of identified acquired ARGs (e.g., on a plasmid, within a transposon, etc.), we identified genes associated with mobile genetic elements on each ARG-carrying scaffold using the INTEGRALL (115), ISfinder (116), PlasmidFinder (117), and NCBI nucleotide (https://www.ncbi.nlm.nih.gov/nucleotide/) databases.

**Statistical analyses.** To investigate whether any host traits influenced the risk of carrying antibiotic-resistant *E. coli*, we performed univariable and multivariable logistic regression models using Firth's penalized likelihood approach implemented in the R package *logistf* (118–120). This method corrects for biases in maximum likelihood estimation due to rare events and small sample sizes while still producing finite and consistent parameter estimates (118, 119). The dependent variable (0/1) was whether an individual carried at least one *E. coli* isolate resistant to at least one antibiotic (i.e., AMR risk). Covariates included age class, sex, home range size, river side (west versus east), social and spatial degree, and social and spatial betweenness. River side was included due to different management strategies for cattle on each side of the river, as well as because previous research has shown that *E. coli* population structure is in part influenced by the river (121). In a separate univariable logistic regression, we also tested for a relationship between AMR risk and the total number of isolates collected per giraffe to confirm that more isolates did not increase the likelihood of detecting antibiotic-resistant *E. coli* ($P = 0.49$). Due to the nonindependence of network data, univariable and multivariable regression *P* values were calculated via permutation methods where the dependent variable was randomized relative to the covariates (10,000 total permutations) (122, 123). *P* values were defined as the proportion of random permutations that yielded a coefficient as extreme as the observed value. Covariates with *P* values of <0.2 in univariable regressions were included in multivariable models. We used forward selection for inclusion of covariates to determine the best-fit multivariate models. To account for potential collinearity among covariates, we calculated generalized variance inflation factor (GVIF) values for each multivariable model and rejected models exhibiting GVIFs of >2.0 (124). GVIFs are recommended over traditional variance inflation factor values when any covariate in the model has more than 1 degree of freedom (125). Candidate models were compared using Akaike's information criterion corrected for small sample size (AICc) (126). Models with a ΔAICc of <2.0 were considered equally parsimonious (126). All analyses were conducted in R v 3.4.2.

To determine whether giraffe social or spatial association networks represent potential transmission pathways for antimicrobial-resistant *E. coli* between individuals, we used the network *k*-test procedure implemented in R (43). This method involves calculating the mean number of infected individuals occurring within one step of an infected individual in a network (i.e., mean infected degree), called the *k*-statistic. The network location of infected individuals is then randomized (node-label swap) and the *k*-statistic is recalculated after each data permutation (10,000 total permutations). The *P* value is calculated by comparing the observed *k*-statistic to the distribution of null *k*-statistics. If the mean number of infected nodes within one step is significantly greater than expected if cases were randomly distributed in the network, this suggests that the pattern of infection cases may have resulted from transmission via network links. Due to the large number of connections between individuals in both the social and spatial networks (many of which had low weight and probably represent transient interactions) and because the *k*-test does not account for link weight, pairs of giraffe were connected only if the link weight (i.e., AS or home range overlap) was greater than the median weight of all network pairs. For weighted networks, we also performed an extension of the *k*-test procedure that uses the average weighted inverse path-length between each infected node and the nearest other infected node (which we term the "path test"), with the assumption that infected nodes will be closer together (shorter path lengths) than random expectations if the network represents potential transmission pathways. For this study, we defined an infected case as a giraffe harboring an *E. coli* isolate with both an ARG-positive scaffold and the corresponding resistance phenotype. Separate *k*-tests and path tests were conducted on both social and spatial networks for each scaffold group that was identified in at least two AMR-positive

giraffe. To correct for multiple hypothesis testing on the same association network, we applied Bonferroni adjustments to all *k*-test and path test *P* values within each network.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02136-18.

**SUPPLEMENTAL FILE 1**, PDF file, 0.04 MB.

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