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Differential Overlap in Human and Animal Fecal Microbiomes and Resistomes in Rural versus Urban Bangladesh

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ABSTRACT Low- and middle-income countries (LMICs) bear the largest mortality burden of antibiotic-resistant infections. Small-scale animal production and free-roaming domestic animals are common in many LMICs, yet data on zoonotic exchange of gut bacteria and antibiotic resistance genes (ARGs) in low-income communities are sparse. Differences between rural and urban communities with regard to population density, antibiotic use, and cohabitation with animals likely influence the frequency of transmission of gut bacterial communities and ARGs between humans and animals. Here, we determined the similarity in gut microbiomes, using 16S rRNA gene amplicon sequencing, and resistomes, using long-read metagenomics, between humans, chickens, and goats in a rural community compared to an urban community in Bangladesh. Gut microbiomes were more similar between humans and chickens in the rural (where cohabitation is more common) than the urban community, but there was no difference for humans and goats in the rural versus the urban community. Human and goat resistomes were more similar in the urban community, and ARG abundance was higher in urban animals than rural animals. We identified substantial overlap of ARG alleles in humans and animals in both settings. Humans and chickens had more overlapping ARG alleles than humans and goats. All fecal hosts from the urban community and rural humans carried ARGs on chromosomal contigs classified as potentially pathogenic bacteria, including *Escherichia coli*, *Campylobacter jejuni*, *Clostridioides difficile*, and *Klebsiella pneumoniae*. These findings provide insight into the breadth of ARGs circulating within human and animal populations in a rural compared to urban community in Bangladesh.

IMPORTANCE While the development of antibiotic resistance in animal gut microbiomes and subsequent transmission to humans has been demonstrated in intensive farming environments and high-income countries, evidence of zoonotic exchange of antibiotic resistance in LMIC communities is lacking. This research provides genomic evidence of overlap of antibiotic resistance genes between humans and animals, especially in urban communities, and highlights chickens as important reservoirs of antibiotic resistance. Chicken and human gut microbiomes were more similar in rural Bangladesh, where cohabitation is more common. Incorporation of long-read metagenomics enabled characterization of bacterial hosts of resistance genes, which has not been possible in previous culture-independent studies using only short-read sequencing. These findings highlight the importance of developing strategies for combatting antibiotic resistance that account for chickens being reservoirs of ARGs in community environments, especially in urban areas.

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In low- and middle-income countries (LMICs), animal husbandry is an important source of household income and nutrition (1, 2). Human exposure to livestock, particularly through cohabitation, is associated with diarrheal disease and impaired child growth (3, 4). Sixty-nine percent (20/29) of studies included in a systematic review and meta-analysis found a significant positive association between human exposures to domestic animals and diarrhea (3). An observational analysis in rural Ethiopia determined that corralling poultry inside households overnight was associated with a 0.25 decrease (standard error = 0.118) in child height-for-age *Z* scores (4). Domestic animals are confirmed reservoirs of enteropathogens, including pathogenic *Escherichia coli*, *Campylobacter* spp., and *Salmonella* spp. (5–7). Living in close proximity to animals, as is common in LMICs, is associated with increased presence of animal feces, which can harbor enteropathogens, in households (8–10). Host-specific fecal markers from animals (dogs, birds, and ruminants) were detected in multiple household reservoirs (soil, hands, and stored drinking water) in rural and urban Bangladesh and rural India (10–12). In rural Bangladesh, increased concentration of an animal-specific fecal marker, BacCow, was associated with increased prevalence of Shiga toxin-producing *E. coli* on hands (11).

Cohabitation between humans and domestic animals may also promote dissemination of antibiotic resistance. Globally, by 2050, more than 10 million deaths due to antibiotic-resistant infections are estimated to occur annually (13). LMICs are predicted to bear the largest burden of these deaths, partially from poor regulation of antibiotic use in humans and animals, management of human and animal excreta, and access to safe drinking water, sanitation infrastructure, and hygiene (13). Antibiotic resistance arises through antibiotic use, accelerated by overuse and misuse of antibiotics (e.g., prophylaxis, incomplete treatment courses, and growth promotion) in humans and domestic animals, which contributes to the selection of intestinal bacterial strains that have acquired resistance (14). Further, in microbial communities, such as the intestinal microbiota, bacteria can exchange antibiotic resistance genes (ARGs) through horizontal gene transfer, allowing the exchange of ARGs between benign bacteria and pathogens, as well as between distinct microbial communities (humans, animals, and environmental matrices) (15, 16). The proliferation of antibiotic resistance in animal gut microbiomes in response to antibiotic use as prophylaxis or growth promotion and subsequent transmission to humans, particularly farm workers, has been demonstrated in intensive farming environments and high-income countries (17). Particularly in LMICs, data on antibiotic-resistant infections acquired in domestic environments, however, are limited. A study among six LMICs—Bangladesh, Bolivia, Ghana, India, Pakistan, and South Africa—found that more than half of community-acquired infections in children were resistant to antibiotics (18). It is crucial to determine whether domestic animals raised through small-scale animal production and backyard farming are important reservoirs of antibiotic resistance in LMICs to determine if community-level interventions in addition to national-level regulation of antibiotic use in agriculture, veterinary medicine, and human medicine could reduce the burden of antibiotic-resistant infections.

While less than 30% of urban Bangladeshi households own domestic animals, compared to more than 70% of rural households, there is a greater ratio of backyard farms ($<$2.5 acres of land) to medium- and large-scale farms in urban than rural communities (19, 20). Although humans might be exposed to a greater number of animals in rural areas, limited space in urban areas makes it more difficult to separate animals and animal feces from humans living in the community. It is unclear how these differing levels of human exposure to animals might affect similarity in gut bacterial communities or intestinal resistomes (collections of all ARGs) between humans and animals in rural versus urban communities. Urban communities have been hypothesized as potential hot spots of antibiotic resistance transmission (21). Previous work has shown that there is a higher proportion of antibiotic-resistant infections among children in urban than rural

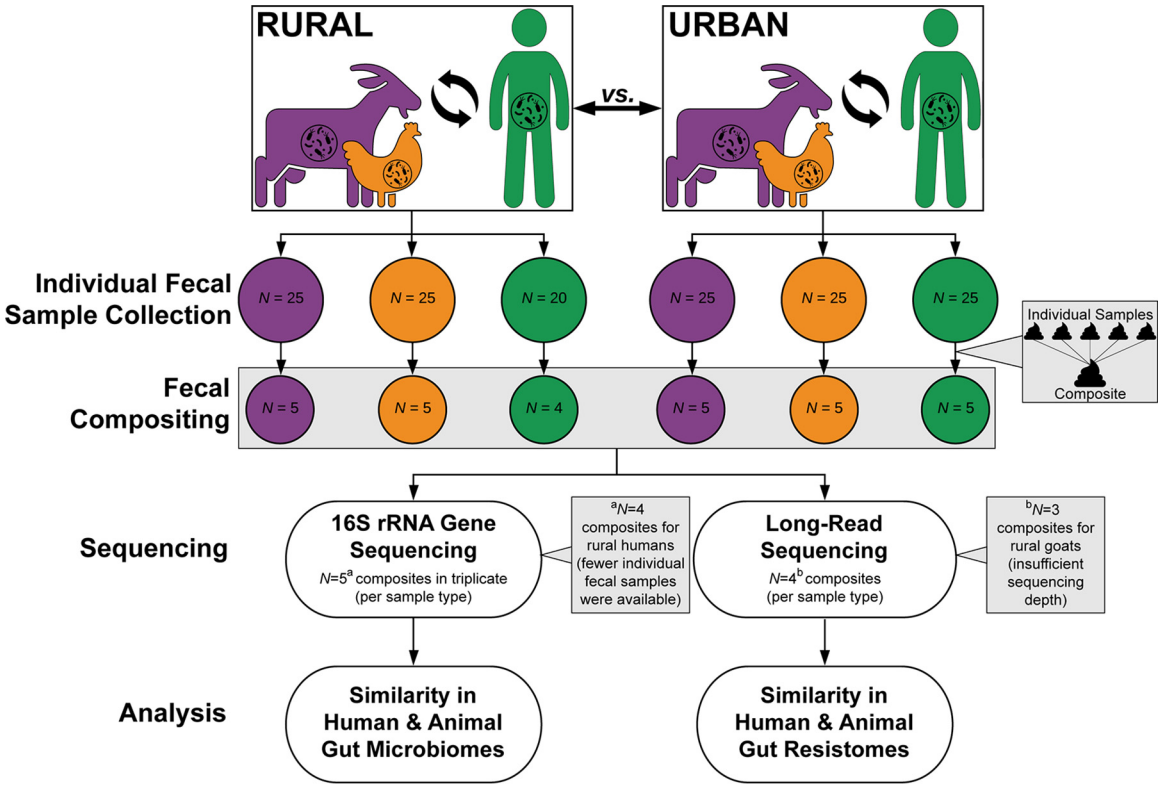

FIG 1 Study design flowchart for comparing human and animal fecal composites in a rural and an urban community.

communities (22–24). As rural residents continue to migrate to urban areas, urban populations are expected to rise (25, 26). Thus, it is increasingly important to characterize transmission pathways of antibiotic resistance, and how they differ in rural versus urban settings, to design targeted and effective interventions.

While prior research in LMICs demonstrates the potential for gut bacteria and ARGs to be shared between humans and domestic animals, metagenomic studies examining resistomes have primarily relied on fragmented assemblies produced with short-read sequencing technologies, limiting their ability to link ARGs to specific bacterial species or locate ARGs on chromosomes or mobile genetic elements (e.g., plasmids and transposons). A study in rural Kenya found greater similarities in gut microbiota composition between children and cattle sharing a household than those from different households, though the results were not statistically significant (27). A study in rural and periurban Peru found that 41% of antibiotic resistance proteins shared by human, animal, and environmental samples were encoded in the same genetic context (neighboring core genes), indicating past horizontal gene transfer, though the short-read assemblies revealed that 11% of unique antibiotic resistance proteins were encoded in multiple contexts (28). Recent work, limited to the study of *E. coli* cultured from children and domestic animals in Ecuador, has shown that while clonal relationships exist, horizontal gene transfer via diverse plasmids may present a greater challenge in the dissemination of antibiotic resistance (29, 30).

Technological advances in molecular methods, such as long-read sequencing, enable improved assemblies of complex metagenomes over those produced with short-read technologies, thus facilitating a deeper understanding of how antibiotic resistance circulates in communities. In this study, we used a combination of 16S rRNA gene amplicon and long-read sequencing to characterize differential overlap in fecal microbiomes and resistomes between humans and common domestic animals (chickens and goats) in a rural versus urban community in Bangladesh (Fig. 1). We also compared ARG abundance by fecal host type and urbanicity and identified potentially pathogenic bacterial species and plasmids carrying ARGs.

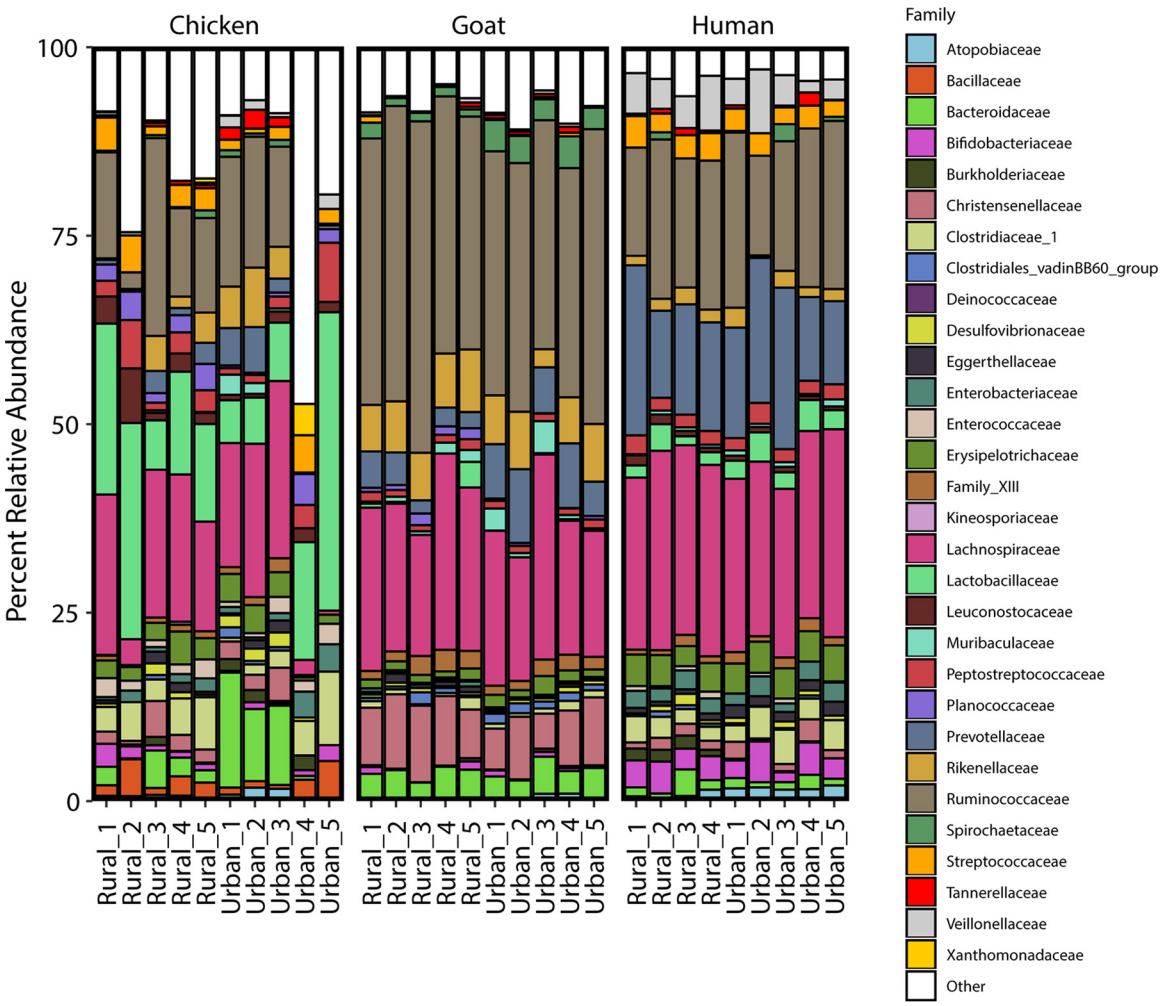

FIG 2 Relative abundance of the 30 most abundant families in all samples from 16S rRNA gene analysis. All other taxa are grouped into the category "Other."

RESULTS

Similarity in gut microbiomes. We obtained a total of 11.7 million reads from the 16S rRNA gene amplicon sequencing of DNA extracts from chicken, goat, and human fecal composites. The average sequencing depths per sample were 97,000 reads in chickens, 134,000 in goats, 144,000 in humans, 72,000 in the positive control (*E. coli* K-12 [ATCC 10798]), 180 in the extraction blank, and 6,600 in the PCR no-template control (nuclease-free water in place of template DNA). On average, 79% of the input reads remained after quality filtering, merging of forward and reverse reads, and chimera removal. Sixty-six percent and 8% of reads were classified at the genus and species levels, respectively. In total, 1,528 amplicon sequence variants (ASVs) were present in chicken fecal composites, 3,270 in goat composites, and 655 in human composites. The relative abundance of the 30 most abundant families in all sample types are presented in Fig. 2. There were 140 ASVs identified in the no-template control (see Fig. S1 in the supplemental material) indicating potential contamination during sample processing. We performed a sensitivity analysis in which the ASVs present in the no-template control were removed from all samples (Fig. S2). Due to the absence of standardized methods to account for negative controls and our finding that the primary study conclusions remained unchanged in the sensitivity analysis, we did not adjust fecal sample ASV counts in the analysis presented here.

The bacterial community compositions of human, chicken, and goat feces were significantly different by fecal host (pairwise permutational multivariate analysis of

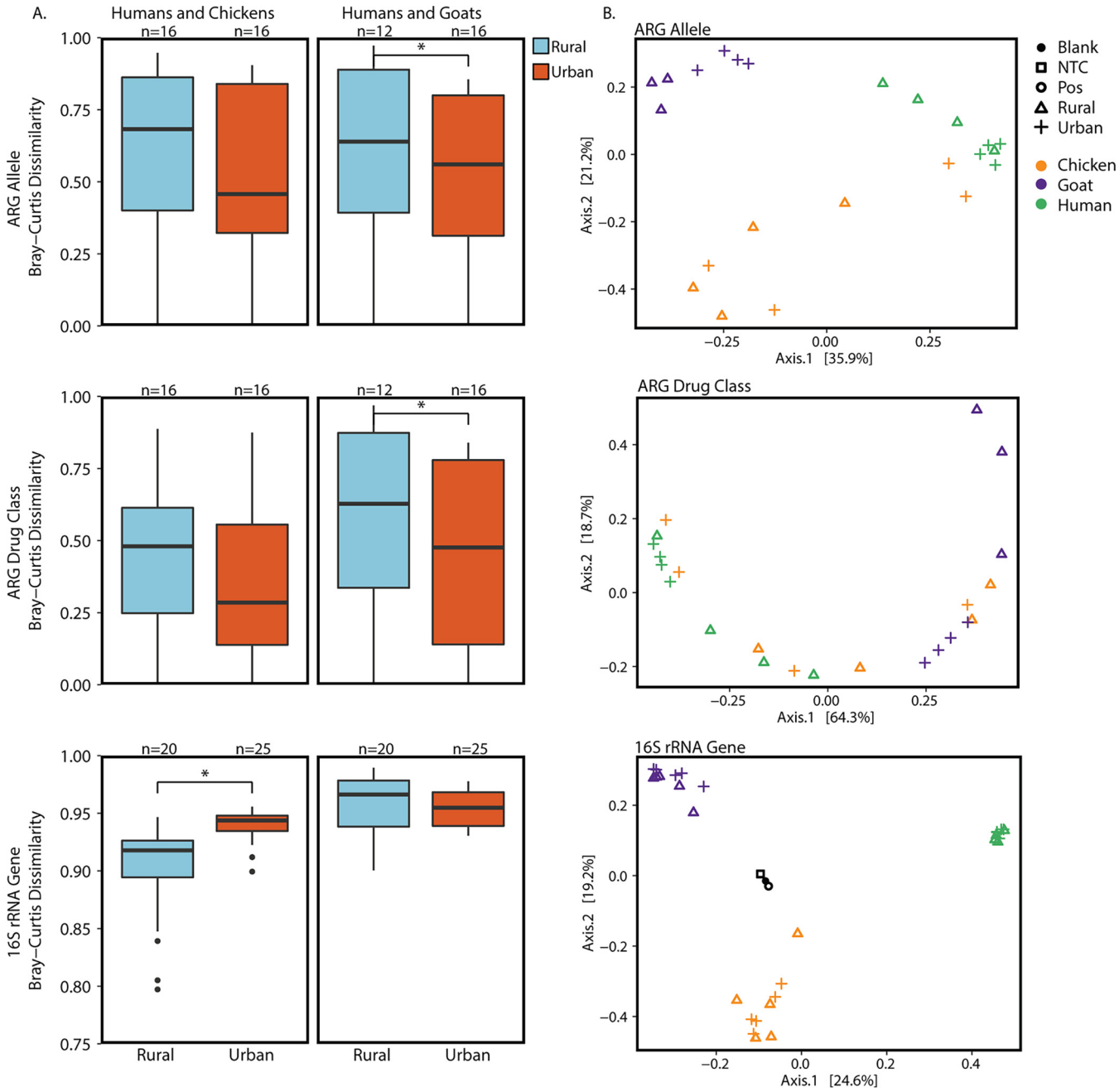

FIG 3 Bray-Curtis dissimilarity between resistomes (top and middle; long-read sequencing) and bacterial community compositions (bottom; 16S rRNA gene analysis) in humans, chickens, and goats. (A) Box-and-whisker plots illustrating Bray-Curtis dissimilarity between humans and chickens (left) and humans and goats (right). The line through each box denotes the median Bray-Curtis dissimilarity index, the lower and upper box boundaries denote the first and third quartiles, respectively, and the whiskers extend to the minimum and maximum indices up to 1.5 times the interquartile range, with outliers plotted as dots. A Bray-Curtis dissimilarity index of 0.00 indicates identical community compositions, and an index closer to 1.00 indicates more dissimilarity between community compositions. (B) Principal-coordinate analyses of Bray-Curtis dissimilarity. Colors indicate fecal hosts, and symbols indicate rural, urban, and control samples. NTC, no-template control. *, significant at a significance level of 0.05 using a Wilcoxon rank sum test.

variance [PERMANOVA]: chicken versus human, R^2 = 0.46; chicken versus goat, R^2 = 0.34; human versus goat, R^2 = 0.50; adjusted P = 0.003 for all) (Fig. 3B). Urbanicity was an important determinant for the gut bacterial community composition in humans and in goats but not in chickens. Specifically, within each fecal host, rural and urban bacterial community compositions were significantly different in goats (PERMANOVA: R^2 = 0.24; P = 0.01) and humans (R^2 = 0.20; P = 0.02) but not in chickens (R^2 = 0.14; P = 0.19). While microbiomes of humans and animals were distinct, human

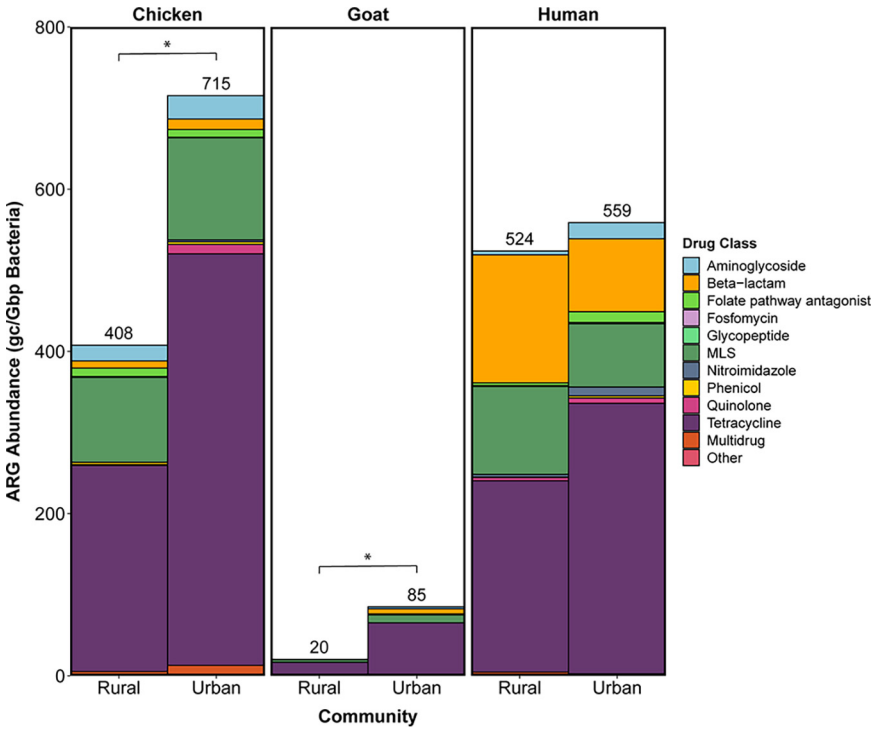

FIG 4 Abundance of ARGs (identified in long-read sequencing data) as gene copies (gc) normalized by gigabase pairs of data classified as bacteria for each fecal host by urbanicity. *, significant at a significance level of 0.05 using a Wilcoxon rank sum test.

microbiomes were more similar to those in chickens than in goats (Wilcoxon rank sum for human-chicken versus human-goat microbiomes, $P < 0.001$). The similarity of human and chicken microbiomes was greater in rural than urban fecal samples (median Bray-Curtis dissimilarity index [interquartile range {IQR}]: rural = 0.92 [0.03], urban = 0.94 [0.01]; Wilcoxon rank sum $P < 0.001$) (Fig. 3A). The similarity between human and goat fecal bacterial community compositions, however, did not differ in the rural versus urban community (rural = 0.97 [0.04], urban = 0.95 [0.03]; Wilcoxon rank sum $P = 0.30$).

Similarity and overlap in resistomes. The long-read sequencing of DNA extracts from the fecal composites yielded, on average, 8.8 million reads per sample type (fecal host by urbanicity) with an average read length of 1,872 bp (standard deviation: 2,546) (Table S1). We obtained a total of 99 Gbp of data, 71% of which was classified as bacteria. Assembly yielded an N_{50} of 50 kbp, on average, and a median contig length of 25 kbp.

We identified a total of 44,077 ARG hits and 497 distinct ARG alleles. Tetracycline, beta-lactam, and macrolide, lincosamide, and streptogramin B (MLS) resistance genes dominated human and animal resistomes (Fig. 4). ARGs were widespread, especially in chicken and human feces. The abundance of ARGs (gene copies normalized by gigabase pairs of data classified as bacteria for each fecal host) was greater in chickens (Wilcoxon rank sum test: rural, $P = 0.034$; urban, $P = 0.021$) and in humans (Wilcoxon rank sum test: rural, $P = 0.034$; urban, $P = 0.021$) than in goats (Table S2). The abundance of ARGs was higher in urban than rural animals, but not significantly different by urbanicity for human guts. Specifically, within each fecal host, ARG abundance (paired by drug class) was greater in urban than rural samples, though results were statistically significant only for chickens and goats (Wilcoxon signed-rank test: chickens, $P = 0.016$; goats, $P = 0.017$; humans, $P = 0.40$) (Fig. 4; Table S3). Similarity between chicken hosts' resistomes did not differ by urbanicity, though between-goat and between-human resistomes were more similar in the urban than rural community (Fig. S3; Table S4). In

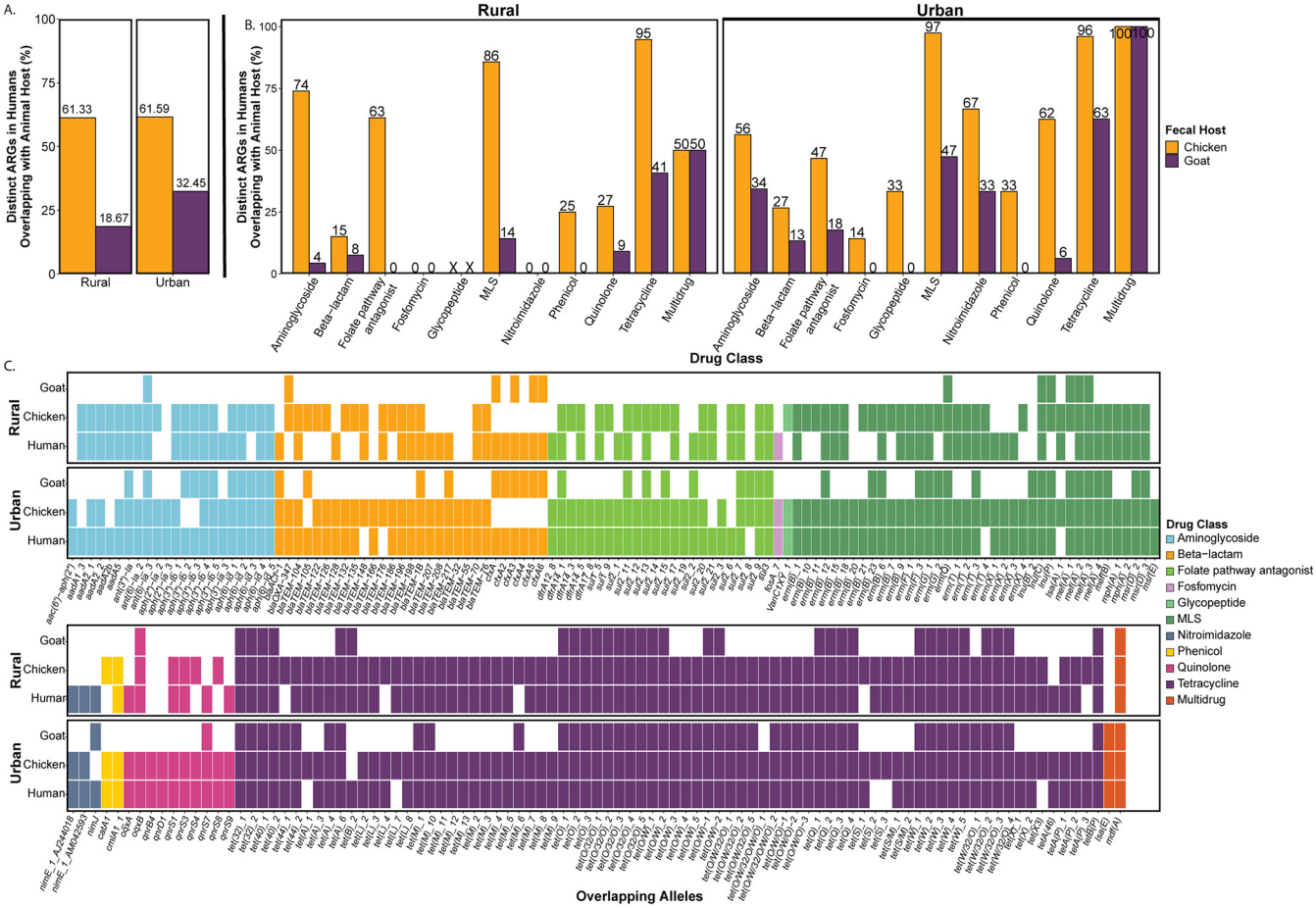

FIG 5 Distinct ARG alleles overlapping between humans and each animal host. (A) Percentage of distinct ARGs identified (using long-read sequencing) in humans that were also found in each animal host. (B) Percentage of distinct ARGs, by drug class, identified (using long-read sequencing) in humans that were also found in each animal host. An "X" indicates that ARGs from that drug class were not detected in humans from that community. (C) ARG alleles overlapping between at least two fecal hosts living in the same community.

the rural versus urban community, the similarity between human and chicken resistomes at the allele level (median Bray-Curtis dissimilarity index [IQR]: rural = 0.68 [0.46], urban = 0.46 [0.52]; Wilcoxon rank sum $P = 0.09$) and grouped by drug class (rural = 0.48 [0.37], urban = 0.29 [0.42]; Wilcoxon rank sum $P = 0.07$) did not differ (Fig. 3A). Human and goat resistomes, however, were more similar in the urban than the rural community at the allele level (rural = 0.64 [0.45], urban = 0.56 [0.49]; $P = 0.01$) and grouped by drug class (rural = 0.63 [0.54], urban = 0.48 [0.64]; $P = 0.02$) (Fig. 3A).

Overlap of distinct ARG alleles was greater between humans and goats (Z-test $P < 0.001$) in the urban than the rural community, though it was not significantly different between humans and chickens (Z-test $P = 0.95$) in urban versus rural samples (Fig. 5A). In the rural and urban communities, the overlap of ARGs in humans and chickens was greater than that in humans and goats (Z-test $P < 0.001$). Among genes overlapping between urban chickens and urban humans, three extended-spectrum beta-lactamase (ESBL) genes, $bla_{\text{TEM-126}}$, $bla_{\text{TEM-132}}$, and $bla_{\text{TEM-207}}$, and three beta-lactamase inhibitor (BLI) resistance genes, $bla_{\text{TEM-32}}$, $bla_{\text{TEM-76}}$, and $bla_{\text{TEM-122}}$, were identified (Fig. 5C). In addition, $bla_{\text{TEM-76}}$ overlapped in rural chickens and rural humans. While not overlapping between fecal hosts living in the same community, additional ESBL genes, $bla_{\text{SHV-12}}$ and $bla_{\text{TEM-147}}$, were detected in chicken and human hosts.

Contigs from the urban chicken, rural human, and urban human samples were classified as plasmids using the MOB-recon tool (Table 1) (31). One plasmid cluster was shared by humans and chickens, but it could not be typed. A total of seven unique plasmid types

TABLE 1 Plasmids carrying antibiotic resistance genes[a]

Fecal host	Cluster ID	Replicon type(s)	ARG allele(s) carried by plasmid	ARG drug class(es)
Rural human	545	IncFII	$bla_{TEM-105}$, *tet*(A)_6	Beta-lactam, tetracycline
	545	IncFII, IncFIIA	bla_{TEM-1B}, *mph*(A)_2	Beta-lactam, MLS
	3619	rep_cluster_1079	*nimE*_1_AM042593	Nitroimidazole
	Novel	rep_cluster_475	*tet*(O/32/O)_4	Tetracycline
	Novel	rep_cluster_1085	*erm*(F)_3	MLS
Urban human	156	NA	*qnrS1*	Quinolone
	487	NA	*tet*(B)_2	Tetracycline
	730	rep_cluster_1418	*aph(6)-Id*_1, bla_{TEM-1B}, *mef*(B), *qnrS1*, *sul3*	Aminoglycoside, beta-lactam, folate pathway antagonist, MLS, quinolone
	1095	NA	*aadA2*_1, *dfrA12*_8, *mph*(A)_1, *sul1*_5	Aminoglycoside, folate pathway antagonist, MLS
	1222	IncFIB, IncR	*ant(3″)-Ia*, *aph(3″)-Ib*_5, *aph(6)-Id*_4, *dfrA14*_1, *qnrS1*, *sul2*_2	Aminoglycoside, folate pathway antagonist, quinolone
	1423	NA	*tetA*(P)_1, *tetB*(P)	Tetracycline
	1552	IncFIIA, IncFII, IncFIB	*erm*(B)_18, *mph*(A)_1	MLS
	1553	NA	*aadA2*_1, *ant(3″)-Ia*, *dfrA12*_8, *mef*(B), *sul3*	Aminoglycoside, folate pathway antagonist, MLS
	1553	NA	*catA1*	Phenicol
	1553	ColRNAI_rep_cluster_1987	bla_{TEM-1B}, *tet*(A)_6	Beta-lactam, tetracycline
	3393	NA	*erm*(B)_18	MLS
	3619	rep_cluster_1079	*nimE*_1_AM042593	Nitroimidazole
	3619	rep_cluster_1079	*erm*(F)_3, *tet*(X)_2	MLS, tetracycline
	Novel	NA	*aac(3)-IId*, *aadA2*_1, *ant(3″)-Ia*, bla_{TEM-1B}, *dfrA1*_9, *mph*(A)_2, *sul1*_5, *tet*(A)_6	Aminoglycoside, beta-lactam, folate pathway antagonist, MLS, tetracycline
	Novel	IncFIIA, IncFII	*aac(3)-IId*, bla_{TEM-1B}	Aminoglycoside, beta-lactam
	Novel	IncQ1	*aadA1*_3, *catA1*, *sul2*_3, *tet*(A)_6	Aminoglycoside, folate pathway antagonist, phenicol, tetracycline
	Novel	rep_cluster_1079	*erm*(F)_1, *erm*(F)_3, *tet*(X)_2	MLS, tetracycline
	Novel	Inc18	*aac(6′)-aph(2″)*, *erm*(B)_23, *str*_1, *tet*(L)_2, *tet*(O/W/32/O)_2	Aminoglycoside, MLS, tetracycline
Urban chicken	2351	NA	*tet*(L)_8	Tetracycline
	3619	rep_cluster_1079	*erm*(F)_3, *tet*(X)_2	MLS, tetracycline
	Novel	rep_cluster_1079	*erm*(F)_3, *nimE*_1_AM042593, *tet*(X)_2	MLS, nitroimidazole, tetracycline
	Novel	rep_cluster_54	*tet*(L)_8, *tet*(M)_4, *tet*(W)_4	Tetracycline

[a]Some fecal hosts (rural chicken, rural goat, and urban goat) are not shown, because no plasmids carrying antibiotic resistance genes were detected in their respective samples. NA, not applicable.

(ColRNAI, Inc18, IncFIB, IncR, IncFII, IncFIIA, and IncQ1) were present in the rural and urban human samples, and none of the plasmids identified in urban chicken samples could be typed. bla_{TEM} genes conferring resistance to beta-lactams were carried by IncF-type and ColRNAI plasmids in human stool.

Of the contigs assembled from the long reads, 87.5% (99,823/114,093) were classified as bacteria. Eighty-nine percent (89,228/99,823) of the bacterial contigs were classified to the strain, subspecies, or species level, of which 2.37% (2,112/89,228) were classified as potentially pathogenic species highlighted in the CDC's 2019 report on antibiotic resistance threats (32). We determined that several important potentially pathogenic bacteria were likely present and carried ARGs, particularly in the urban community (Fig. 6). Most (95.7%, 45/47) of the ARG-carrying contigs aligning to potential pathogen reference sequences were also determined to be chromosomal using the MOB-recon tool, and two ARG-carrying contigs aligning to potential pathogens were classified as plasmids: (i) a *Campylobacter jejuni*-aligning contig carrying *tet*(O/32/O) in a rural human sample and (ii) an *E. coli*-aligning contig carrying *qnrS1*, *sul3*, *aph(6)-Id*, bla_{TEM-1B}, and *mef*(B) in an urban human sample.

In total, 29 distinct ARGs, representing 10 drug classes (aminoglycoside; beta-lactam; fluoroquinolone; folate pathway antagonist; fosfomycin; macrolide, lincosamide, and streptogramin B; phenicol; quinolone; rifampicin; and tetracycline), were carried by potential pathogens. No ARGs were carried by potential pathogens in rural chickens or rural goats. Of the six bacterial species carrying ARGs in humans, two were also present in urban animal hosts.

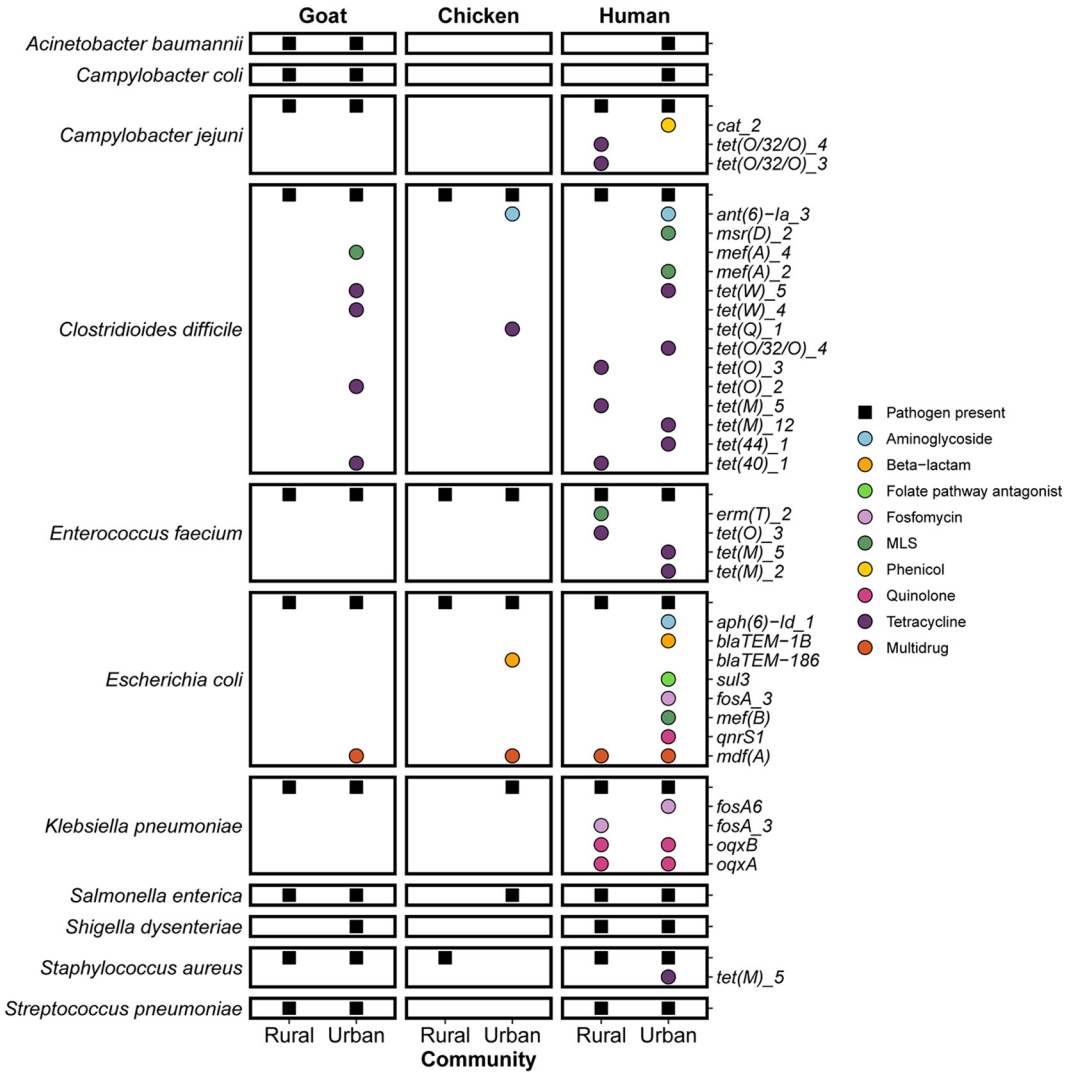

FIG 6 Prevalence of potentially pathogenic bacteria species and those carrying ARGs identified in contigs assembled from long reads. Black squares indicate that at least one contig for the respective fecal host was classified as the bacterial species on the left. Colored dots indicate ARGs (by drug class) identified on contigs classified as the bacterial species listed on the left. Bacterial species represent those identified in the CDC's 2019 report on antibiotic resistance threats in the United States (31).

Clostridioides difficile organisms carrying tetracycline [*tet*(W), *tet*(O), *tet*(40)] and macrolide [*mef*(A)] resistance genes were present in humans and urban goats, and *C. difficile* organisms carrying aminoglycoside [*ant(6)-Ia*] resistance genes were present in urban humans and urban chickens. Multidrug [*mdf*(A)] resistance genes carried by *E. coli* were prevalent in humans, urban chickens, and urban goats. *E. coli* isolates carrying beta-lactam (bla_{TEM}) resistance genes were identified in urban chickens and urban humans. Only three contigs carrying ARGs were classified as potential pathogens to the strain or subspecies level. *E. coli PCN033*, carrying a multidrug [*mdf*(A)] resistance gene, was identified in a rural human sample, and *C. difficile M120*, carrying macrolide [*mef*(A)] and tetracycline [*tet*(44)] resistance genes, were present in an urban goat and an urban human sample, respectively. Though the potential pathogen classifications were confirmed through BLASTN with low *E*-values and high percent identity (Table S5), we noted off-target hits with similar *E*-values and percent identities for the *E. coli PCN033* and *C. difficile M120* classifications.

DISCUSSION

We found greater similarity in bacterial communities between humans and chickens in a rural than an urban community in Bangladesh, though human and animal microbiomes

were generally distinct (Bray-Curtis dissimilarity indices $>$ 0.93) in both communities. Human-chicken similarities in the rural community could partially be explained by a higher prevalence of animal ownership in the rural site. While 94% of households owned livestock in the rural community, only 52% owned animals in Dhaka, Bangladesh (8, 19). As our study did not specifically collect human stool from animal-owning households, it is possible that human participants from the urban community had limited exposure to animals. Future research purposely sampling human stool from animal-owning households may help elucidate whether rearing animals nearer to human living quarters impacts human-animal microbiome similarities. Additionally, goats are ruminants, which have complex digestive systems that make their gut microbiomes diverse and highly dissimilar from those of nonruminants (33, 34). Further, human microbiomes are generally more diverse in urban than rural areas (35). Findings from previous studies have shown that gut microbiotas are influenced by geography and diet (28, 35–38). People living in urban Bangladesh tend to have diverse diets consisting of most food groups (i.e., starches, pulses, fish, eggs, meat, vegetables, fruit, milks, oils, spices, sweets, and beverages), while those in rural areas are more likely to have limited diets (39). As free-roaming animals are common in Bangladesh, similar human and chicken gut microbiomes in the rural community might be explained by chickens scavenging through human rubbish and/or being fed scraps and leftovers from human meals (8, 40, 41). It is also possible that the differences in bacterial communities between rural and urban goats could be attributed to grazing on differing food sources in the rural and urban communities.

On the other hand, there was greater similarity in resistomes between humans and goats (and within goats and within humans) in an urban versus a rural community in Bangladesh. Urban settings might serve as hot spots of zoonotic antibiotic resistance transmission, partially due to poor human and animal fecal management (21). In Dhaka, Bangladesh, for example, an estimated 2% of human fecal waste is safely managed while the remaining 98% directly enters the environment (42). There is therefore ample opportunity for grazing animals to consume food and water contaminated with human feces.

Our findings highlight domestic chickens as important reservoirs of antibiotic resistance. Similarly, a comparison of resistomes in chickens, pigs, and humans in China found ARG abundance in chicken feces to be the highest (43). Another study found that publicly available human stool bacterial genomes shared a greater number of mobile ARGs with chicken gut microbiomes than with pig and cattle microbiomes (44). The high abundance of ARGs in chicken feces and resistome sharing between chickens and humans is consistent with existing literature, much of which has focused on occupational exposures in commercial settings. For example, recent work in Nigeria identified occupational exposure to chickens as a risk factor for multidrug-resistant *E. coli* colonization among poultry workers and identified similar resistance patterns and identical plasmid replicons in *E. coli* recovered from chickens and poultry workers (45–47). While previous work in Ecuador found that higher levels of antibiotic-resistant bacteria were isolated from commercially raised poultry than backyard poultry, our work uniquely demonstrates the important role of poultry in antibiotic resistance circulation in an urban community (48).

Tetracycline resistance genes dominated the resistomes of humans, chickens, and goats. Tetracyclines have been widely used for prophylaxis and to treat a variety of Gram-positive and Gram-negative bacterial infections in humans and domestic animals; tetracyclines have also been used as growth promoters in animal husbandry (49). Our results mirror previous work that found tetracycline resistance genes to be among the most abundant ARGs in human and animal feces (43, 50–52). In layer farms in Bangladesh, tetracycline was one of the most prevalent antibiotic residues, in addition to ciprofloxacin and enrofloxacin (fluoroquinolones), in the eggs, tissue, and internal organs of chickens (53, 54). Genes conferring resistance to MLSs were also abundant in our study, particularly in humans and chickens. Similar to tetracyclines, MLSs have a long history of prophylactic and treatment use in human and veterinary medicine (55). Additionally, beta-lactam resistance genes, including ESBL and BLI resistance genes,

were prevalent in our study samples, especially in chickens and humans. Beta-lactams, particularly cephalosporins, were one of the most widely prescribed antibiotics in multiple studies surveying pharmacies, outpatients, and hospitals in rural and urban Bangladesh (56–59). ESBL-producing *Enterobacteriaceae* infections are a global public health concern and have limited treatment options, often requiring last-resort treatment with carbapenems (60). In addition to other Gram-negative bacterial infections, ESBL-producing *Enterobacteriaceae* infections are often treated by coadministering beta-lactams and beta-lactamase inhibitors (61). Further, beta-lactamase enzymes are widely plasmid-mediated, allowing for ease of horizontal gene transfer between bacterial hosts and between humans, animals, and the environment (15, 16, 29, 62). Although human and animal, especially chicken, resistomes were dominated by genes conferring resistance to similar antibiotic classes, it is not possible to assume directionality of exchange. While this is partially due to antibiotic classes being used across both human and veterinary medicine and animal husbandry, widespread human and animal fecal contamination in shared living environments further complicates exchange dynamics. Particularly, when allowing animals to roam and graze freely is common, animals are likely at risk of acquiring ARGs originating from humans through the environment, emphasizing how close proximities between humans and domestic animals propagates antibiotic resistance, regardless of whether humans or animals are the primary sources.

We detected bla_{TEM} genes (beta-lactam resistance) located on long-read assembled contigs classified as IncF plasmids in rural and urban human stool and on a contig classified as a ColRNAI plasmid, a plasmid group known to carry beta-lactam resistance in *Enterobacteriaceae*, in urban human stool (62–66). IncF plasmids are particularly concerning in the spread of antibiotic resistance, as they often carry multiple replicons, or regions of the genome where replication is initiated, allowing rapid evolution and great adaptability to promote acquisition by various bacterial host species (65). Although there is a recognized importance of mobile genetic elements (e.g., plasmids) in horizontal gene transfer and the spread of antibiotic resistance, we detected few plasmids in our long-read sequencing data. Accurate identification of plasmids using the MOB-suite tool, as well as other plasmid detection tools, relies on high quality genome assemblies as input (31). Despite advances in the accuracy of long-read sequencing technology, sequencing errors and insufficient coverage make plasmid detection difficult in complex metagenomes using long reads alone. Deeper sequencing and hybrid assemblies of short- and long-read data may improve the ability to characterize the role of plasmids in human and animal resistomes. Further, MOB-suite uses a database-based approach, and performance is reduced when classifying novel plasmids not available in NCBI's RefSeq and GenBank databases (31). Due to overrepresentation of plasmids from human clinical isolates from high-income countries in databases, it is possible that we were limited in our ability to detect plasmids in chickens and goats or specific to Bangladesh using a database approach.

We leveraged long sequence reads to identify bacterial hosts of ARGs, focusing on species identified as antibiotic resistance threats by the CDC (32). While these species often present as commensals in human and animal guts, some strains are pathogens or opportunistic pathogens. Importantly, ARG-carrying commensals can pose a health risk, as horizontal gene transfer can facilitate exchange of ARGs between benign and pathogenic bacteria in intestinal microbiomes (15, 16). *C. difficile* carried a wide range of ARGs across various drug classes (aminoglycoside, MLS, and tetracycline) in humans and animals. Spore-forming *C. difficile* can colonize the gut without causing illness, particularly in infants; asymptomatic colonization in adults is rare but possible (67–69). Historically, *C. difficile* infections have been known to arise after antibiotics have been taken and account for the highest morbidity and mortality of hospital-acquired infections in high-income countries, though the burden of *C. difficile* infections in LMICs is poorly understood (70–73). There is, however, increasing recognition of *C. difficile* as a community-acquired infection, in addition to hospital-acquired infection and potential zoonosis (74, 75). We also identified *E. coli* carrying bla_{TEM}, a gene family containing

alleles conferring resistance to beta-lactams, in urban chickens and urban humans. Previous work has documented poultry as a reservoir of ESBL-producing *Enterobacteriaceae* (76, 77). Our results therefore stress the importance of understanding and targeting transmission pathways of ESBL-producing *Enterobacteriaceae* in curbing antibiotic resistance, particularly in an urban community where humans and animals live in close proximity and share environments.

This study has some limitations. Because we leveraged human stool collections from previous studies, urban human samples were collected 2 years prior to urban animal samples, while rural human and animal samples were simultaneously collected. Temporal differences in sample collection between the two communities might partially explain the greater microbiome similarity observed between rural humans and chickens compared to urban humans and chickens. Due to this temporal issue, the compositing of fecal samples, and the use of 16S rRNA gene sequencing as opposed to bacterial strain detection, we are unable to draw conclusions about whether zoonotic transmission of gut bacteria or ARGs occurred (or its directionality) in our study communities. As chicken and goat feces were collected from the ground, the findings presented here may reflect fecal host and environmental microbiomes and resistomes; however, trained sample collectors took care to sample only from the center and top of fresh feces piles to limit soil contamination. In addition, few barcodes were detected in the long-read sequence data for one of the rural goat samples, so we were not able to detect any ARGs, plasmids, or potential pathogens in that sample; however, as all long-read sequencing analyses (excluding the Bray-Curtis dissimilarity analysis) relied on pooled data from each fecal host in a particular setting, we do not anticipate that the missing sample influenced the interpretation of our results. Also, we obtained less long sequence reads, as well as more fragmented reads, from chicken DNA extracts than human and goat DNA, potentially due to the heterogeneity, heat treatment (required by our USDA import permit), or high uric acid concentration of chicken fecal matter. It is therefore likely that chicken metagenome coverage was low, biasing plasmid and potential pathogen detection; we do not believe that this limitation drastically impacted ARG detection, as the normalized abundance of ARGs was higher in chickens than in humans and goats. Finally, our results may not be generalizable across all rural and urban areas in Bangladesh or other LMICs, as we sampled only one rural and one urban community; there may be factors other than urbanicity that we were unable to measure, confounding our findings.

This community-level study demonstrates similarities in resistance profiles, as well as revealing potential pathogens carrying resistance genes, in humans and domestic animals living in the same community in rural and urban Bangladesh. Our results contribute to a gap in knowledge regarding the role of small-scale animal production in antibiotic resistance dissemination within resource-constrained communities. While domestic goats and chickens harbored potentially pathogenic hosts of ARGs, our results suggest that compared to goats, backyard poultry might be an especially important reservoir for ARGs that are also present in the human gut. In addition, our findings provide some evidence that ARGs are more widespread in an urban than a rural community. While directionality for ARG exchange between humans and animals in community settings is still unclear, substantial sharing of ARGs in humans and animals in both rural and urban communities in this study highlights the importance of developing interventions and antibiotic stewardship strategies that take into account shared environments between human and animal reservoirs.

MATERIALS AND METHODS

Study sites. We collected human and animal fecal samples from one rural and one urban community in Bangladesh. The rural community, Mymensingh district, is located roughly 120 km north of Dhaka, the capital city of Bangladesh. Rural households in Mymensingh are typically organized into compounds that share a courtyard, sometimes including a water source and/or latrine. Mymensingh compounds are characterized by high levels of animal ownership (94%), poor drinking water quality (77% of stored water samples had detectable *E. coli*), limited latrine ownership (54%), and limited access to handwashing stations with soap (21%) (8, 78). The urban community, Tongi subdistrict, is 15 km north of

Dhaka. It is densely populated, with many families living in single-room homes and sharing a common stove, water source, and/or latrine with up to 15 other families. While less than 30% of urban households own animals, a greater proportion of animal-owning households have less than 2.5 acres of land in urban than in rural areas (9, 19, 20). Similar to rural areas, low-income households in urban Bangladesh are characterized by poor drinking quality (70% of stored water samples had detectable *E. coli* in Tongi) and insufficient sanitation facilities (42, 79). Household asset ownership is higher among Tongi (T) residents than among Mymensingh (M) residents with regard to televisions (T, 84%; M, 51%), mobile phones (T, 94%; M, 86%), wardrobes (T, 63%; M, 17%); and refrigerators (T, 36%; M, 8.0%) (78, 79). According to recent studies, reported household antibiotic use among children under 5 years is fairly similar in rural (39%) and urban (44%) communities in Bangladesh (79, 80).

Sample collection. Between June and August 2015, 50 individual chicken and 50 individual goat fecal samples were identified by observation in public and domestic areas in Bangladesh, half of which were from Mymensingh (rural) and half from Tongi (urban). Using sterile scoops, fresh feces were scooped from the center and top of feces piles to avoid soil contamination. Samples were stored on ice and transported to a field laboratory (Mymensingh) or International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b; Tongi) within 6 h. Trained icddr,b staff carried out the animal fecal sample collection.

Twenty-five human stool samples were collected from adult (>18 years old) residents of different households in Tongi between April and October 2013; participants were asymptomatic members of households of cholera patients presenting with diarrhea to the Tongi Sub-district Hospital or icddr,b Dhaka Hospital (81). The samples were transported in Cary-Blair medium to icddr,b and frozen at −80°C. Between June and September 2015, 20 human stool samples were collected from adult (>18 years old) residents of different households in Mymensingh; participants were primary caregivers of target children enrolled in the parent trial (78). The samples were transported to the field laboratory and frozen at −80°C before being shipped to icddr,b on dry ice. Written informed consent was obtained from all study participants. The studies were approved by the icddr,b Ethical Review Committee (PR-11063 and PR-13004), Johns Hopkins University Institutional Review Board (IRB) (00004795), University of California, Berkeley Committee for the Protection of Human Subjects (2011-09-3652), and Stanford University IRB (25863).

Animal fecal samples were immediately processed after arrival at the laboratories, and human stool samples were thawed from −80°C prior to further processing. The samples were combined into fecal composites for each fecal host (chicken, goat, and human) from the urban or rural community by measuring 0.2 g of five individual samples from corresponding locations into 50-mL centrifuge tubes using ethanol- and flame-sterilized spatulas. Samples were composited due to resource constraints, and this allowed us to reduce the number of samples while preserving information from all collected samples. We used this approach previously when working in resource-constrained environments (9, 82). After the samples were mixed, 1-g composites were transferred to 1.5-mL microcentrifuge tubes. One milliliter of RNAlater solution (Invitrogen, Carlsbad, CA) was mixed into each composite and left to sit for 5 min. Five composites were made for each fecal host in each community (rural and urban), with the exception of rural human stool; as each composite contained equal masses of five individual fecal samples and there were only 20 total rural human stool samples, only four composites were possible for rural human stool. Chicken fecal composites were treated in a hot water bath (100°C) for 20 min in accordance with our U.S. Department of Agriculture (USDA) import permit (128693). All composites were stored at −80°C before shipment at room temperature to Stanford University, where samples were stored at −80°C upon arrival. Aliquots for the 16S rRNA gene analysis were stored at −80°C (stored 2 years and 9 months before processing) at Stanford University. After storage at Stanford University, aliquots for the long-read sequencing analysis were shipped on dry ice to Tufts University and stored at −80°C (stored an additional 4 months before processing).

DNA extraction. For 16S rRNA gene analysis, DNA was extracted from 0.25 g of each fecal composite using the PowerSoil extraction kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. We extracted one blank sample per day of DNA extractions (three extraction controls total) to control for contamination during the extraction process. *E. coli* K-12 (ATCC 10798) was used as a positive control. The culture was grown on tryptic soy agar, and a single colony was suspended in nuclease-free water (Thermo Fisher Scientific, Waltham, MA). The suspension was centrifuged at 10,000 × *g* for 5 min, and the pellet was resuspended in nuclease-free water, heated to 100°C for 10 min, cooled at 20°C for 10 min, and centrifuged at 14,000 × *g* for 5 min. The supernatant was used as the template in PCRs.

Due to budget constraints, only four of the five fecal composites from each fecal host were processed with long-read sequencing. For long-read sequencing, DNA was extracted from 0.25 g of each fecal composite using the DNeasy PowerSoil Pro extraction kit (Qiagen, Germantown, MD). We followed the manufacturer's instructions, with the exceptions of using an alternate lysis method (heating samples at 70°C for 10 min followed by vortexing at full speed for 10 min; adapted from Earth Microbiome Project's DNA extraction protocol) and eluting in Tris-EDTA buffer (Integrated DNA Technologies, Inc., Coralville, IA) to prevent degradation (83). We extracted one blank extraction control per day of DNA extractions (three extraction controls total). DNA purity was quantified using a NanoDrop (Thermo Fisher Scientific, Waltham, MA). For extraction controls, nucleic acid concentrations were measured using a Qubit 4.0 fluorometer (1× dsDNA HS assay kit; Thermo Fisher Scientific, Waltham, MA). For fecal composite extracts, nucleic acid concentrations and fragment size distributions were assessed using a 2100 Bioanalyzer (Agilent, Santa Clara, CA) at the Tufts University Core Facility (Boston, MA). Quality control parameters are presented in Table S6.

16S rRNA gene amplicon generation and sequencing. We used a previously described 16S rRNA gene sequencing Illumina amplicon protocol with 515f and 805r primers (GTGYCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT, respectively) that targeted the V4 region of the 16S rRNA gene (84–

89). Golay barcodes were embedded in each forward primer (Table S7). Previously described PCR protocols were used to generate 16S rRNA gene amplicons from all samples, including the negative and positive controls: 25-μL PCR mixtures consisted of 2× Qiagen HotStarTaq Plus master mix (Germantown, MD), 10 μM forward primer, 10 μM reverse primer, and 1 μL of template DNA. PCRs were run in triplicate using the following thermocycler conditions: 94°C for 3 min; then 35 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 90 s; followed by 72°C for 10 min and a hold at 4°C (84–88). A no-template control reaction was included in every PCR run. After PCR, we visualized product size using electrophoresis on 1.5% agarose gels containing ethidium bromide. All fecal composites and positive controls showed a band of appropriate size (~390 bp). The PCR triplicates each received unique barcodes during PCR (i.e., each sample was sequenced in triplicate). We did not observe any bands in the lanes of the gel containing the negative controls, including extraction and no-template controls.

A Qubit 2.0 fluorometer (dsDNA HS assay kit; Thermo Fisher Scientific, Waltham, MA) was used to quantify the nucleic acid concentration in PCR products. Based on those concentrations, amplicons from the samples and the positive control were multiplexed and pooled in equimolar proportions (10 μM) for sequencing. Additionally, we multiplexed and pooled extraction and no-template controls (negative controls) for sequencing. Ten microliters of each of the negative-control pools was added to the total pool before purification. The total pool was purified using the UltraClean PCR cleanup kit (MoBio, Carlsbad, CA) before sequencing. Sequencing was performed using an Illumina MiSeq at the Stanford Functional Genomics Center (Stanford, CA) (89). We used 250-bp paired-end reads (2 × 250) and spiked the total pool with 10% PhiX before sequencing. We sent index primers (AATGATACGGCGACCACCGAGATCTACACGCT), read 1 primers (TATG GTAATTGTGTGYCAGCMGCCGCGGTAA), and read 2 primers (AGTCAGCCCAGCCGGACTACNVGGGTWTCT AAT) with the total pool (84–88).

Long-read sequencing. Long-read sequences were obtained from DNA extracts using MinION (Oxford Nanopore Technologies [ONT], Oxford, United Kingdom). Library preparation was conducted using the SQK-LSK109 ligation sequencing kit (ONT) according to the manufacturer's instructions. When libraries were prepared for goat and human samples, the L fragment buffer (LFB) was used to enrich for DNA fragments of 3 kbp or longer. Since DNA concentrations were low in chicken fecal composite extracts (Table S1), when libraries were prepared for chicken samples, the S fragment buffer (SFB) was used to retain DNA fragments shorter than 3 kbp. Two fecal composite libraries were multiplexed per flow cell (FLO-MIN106; ONT) in equimolar proportions (except for chicken samples, for which all DNA was retained to account for the lower DNA concentrations) using the EXP-NBD103 native barcoding kit (ONT).

Data processing and analysis. (i) 16S rRNA gene amplicon analysis. The DADA2 pipeline was used to process forward and reverse reads (90). Forward reads were truncated to 173 nucleotides and reverse reads were truncated to 162 nucleotides, after which the quality score dropped. Paired-end reads were merged to yield 250-bp sequences, and chimeras were removed. Taxonomy was assigned in DADA2 using a naive Bayes classifier that was trained on the Silva v132 database (91, 92). Species-level identification was based on 100% identity between the reference database and ASVs (93).

Data were analyzed using phyloseq (version 1.26.1) in R (v3.5.0) (94). All ASVs associated with eukaryotic organelles—chloroplasts and mitochondria—were removed. ASVs were normalized using the inverse hyperbolic sine transformation (95). ASVs identified in the three technical replicates (from the PCR step of library preparation) were merged by summing, and the merged data were used for all subsequent analyses. To determine the similarity of bacterial communities between humans and animals, we calculated pairwise Bray-Curtis dissimilarity indices between humans and goats and between humans and chickens in rural and urban areas. A Wilcoxon rank sum test ($P < 0.05$) was used to determine if the differences in Bray-Curtis indices were statistically significant in urban versus rural areas. Variables contributing to differences between communities, such as fecal host and location, were identified by PERMANOVA or pairwise PERMANOVA with Bray-Curtis dissimilarity using vegan (v2.5.6) and pairwiseAdonis (v0.4) in R (v3.5.0) (96).

To determine if sample processing contamination impacted the results, a *post hoc* sensitivity analysis was conducted where ASVs classified as the 140 taxa present in the no-template control (Fig. S1) were removed from all samples and Bray-Curtis dissimilarities were recalculated.

(ii) Long-read sequencing analysis. Long-read sequences were base called, demultiplexed, and trimmed using ONT's toolkit, Guppy (v3.3.0). To identify ARGs, reads were aligned to the ResFinder database (duplicates removed) using Minimap2 (v2.15) (97, 98). Only primary ARG alignments with ≥90% similarity and ≥100-bp alignment lengths were retained for the analysis. To quantify data classified as bacteria, raw reads were aligned to the National Center for Biotechnology Information (NCBI) RefSeq database (plasmid sequences removed) using a least-common-ancestor approach in Centrifuge (v1.0.4) (99). In accordance with a previously published normalization method, ARG gene copies (sum of alignment length over reference read length for all relevant ARG hits) were normalized by gigabase pairs of data classified as bacteria for each sample type and each ARG (100). Wilcoxon matched-pairs signed-rank tests ($P < 0.05$) were used to test for statistical differences in the normalized abundance of ARGs, paired by drug class, in urban and rural samples by fecal host. Wilcoxon rank sum tests ($P < 0.05$) were used to test for differences in the normalized abundance of ARGs in human, chicken, and goat samples by urbanicity. To test the hypothesis that human and animal resistomes were more similar in the rural than the urban community, Bray-Curtis dissimilarities were calculated for all pairwise comparisons, and a Wilcoxon rank sum test ($P < 0.05$) was used to determine statistical significance. We identified overlapping distinct resistance gene alleles between humans and each animal within the urban community and within the rural community by generating ARG overlap matrices and using two-proportion Z tests ($P < 0.05$) to determine statistical significance in the rural versus urban community.

To identify chromosome- or plasmid-associated ARGs, we determined whether long reads aligning to ARG reference sequences were also classified as potential pathogens or plasmids. Specifically, to

identify potential pathogens, reads were assembled using Flye (v2.6) (with the meta option) and polished using ONT's tool for sequence correction, medaka (v0.11.0) (101). Polished contigs were aligned to the modified RefSeq database using Centrifuge and to the modified ResFinder database using Minimap2 (v2.15) (97–99). We focused our analysis on the 12 bacterial pathogens identified as antibiotic-resistant threats in the CDC's 2019 report on antibiotic resistance threats in the United States: *Acinetobacter baumannii*, *Campylobacter coli*, *Campylobacter jejuni*, *C. difficile*, *Enterococcus faecium*, *E. coli*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Salmonella enterica*, *Shigella dysenteriae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (32). The reliability of classifications at the subspecies and strain levels of pathogens carrying resistance genes was assessed by aligning potential pathogen sequences against their respective classifications in NCBI's RefSeq database using BLASTN; an *E*-value that rounded to 0 (BLASTN uses a double-precision floating-point format; therefore, the smallest *E*-value reported is about 1e−179) and an identity of ≥98% were the thresholds used to confirm whether a pathogen classification was reliable (102). Plasmids were identified from the contigs using the MOB-recon tool with default parameters to determine whether identified ARGs were chromosome or plasmid associated (31). Long-read data from the same type of fecal host and location were pooled for presentation of the results. Statistical analyses and data visualization were conducted using Stata SE (v14.2) and R (v3.6.3).

Data availability. Metagenomic sequence reads and 16S rRNA amplicon sequences are available in the sequence read archive (SRA) under accession numbers SRR13059261 to SRR13059374 and BioProject number PRJNA678365. Human sequences were removed from all samples. Relevant analysis scripts and files are available in GitHub (https://github.com/jennaswa/arg_bd).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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