TITLE: *Escherichia coli* population structure and antibioresistance at a buffalo/cattle interface in southern Africa

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ABSTRACT

At a human/livestock/wildlife interface, *Escherichia coli* populations were used to assess the risk of bacteria and antibioresistance dissemination between hosts. We used phenotypic and genotypic characterization techniques to describe the structure and the level of antibioresistance of *E. coli* commensal populations and the resistant *Enterobacteriaceae* carriage of sympatric African buffalo (*Syncerus caffer caffer*) and cattle populations characterized by their contact patterns in the southern part of Hwange ecosystem in Zimbabwe. Our results 1) confirmed our assumption that buffalo and cattle share similar phylogroup profiles, dominated by B1 (44.5%) and E (29.0%) phylogroups, with some variability in A phylogroup presence (from 1.9 to 12%), 2) identified a significant gradient of antibioresistance from isolated buffalo to buffalo in contact with cattle and cattle populations expressed as the Murray score among *Enterobacteriaceae* (0.146, 0.258, 0.340, respectively) and as the presence of tetracycline, trimethoprim and amoxicillin resistant subdominant *E. coli* strains (0, 5.7 and 38%, respectively); 3) evidenced the dissemination of tetracycline, trimethoprim and amoxicillin resistance genes (*tet, dfrA*, bla_{TEM-1}in 26 isolated sub-dominant *E. coli* strains between nearby buffalo and cattle populations that led us 4) to hypothesize the role of the human/animal interface in the dissemination of genetic material from human to cattle and towards wildlife. The study of antibiotic resistance dissemination in multi-host systems and at anthropised/natural interface is necessary to better understand and mitigate its multiple threats. These results also contribute to attempts aiming at using *E. coli* as a tool for the identification of pathogen transmission pathway in multi-host systems.

KEYWORDS: African buffalo; antibiotic resistance; cattle; disease ecology; *Escherichia coli*; tetracycline; amoxicillin; trimethoprim; wildlife/livestock interface;

INTRODUCTION

As human activities increase, the pressure on natural ecosystems through land encroachment, unsustainable use of natural resources and fragmentation of habitats tend to expand worldwide (1). This trend is exacerbated by human population growth and the need to access more land to feed all in developing countries (2, 3). In these contexts, the spread of pathogens and genetic material can represent a burden on wildlife, livestock and human population health (4-6). Diseases significantly impact livestock productions, which are a key livelihood option in semi-arid areas, and can also threaten endangered wildlife species (7). The dissemination of antibiotic resistance (ABR) into remote, supposedly pristine, areas resulting from a high and inappropriate use of antibiotics in humans and domestic animals (in particular medicated feed) (8, 9), demonstrates how the most remote ecosystems are not exempt of a human footprint (10). The consequences of ABR diffusion in natural ecosystems are largely unknown. However, the evolution and selection of resistance genes in the wild could compromise the use of antibiotics (11), the main tool to fight infectious diseases in domestic animals and human (10). Moreover, the ABR pollution "in the wild" could threaten biodiversity (12).

The dynamics and processes of microorganism transmission between hosts and the environment should therefore be a focus of research at wildlife/livestock/human interfaces to provide management options to reduce or deal with their negative effects (i.e. impact on human health, livestock production and biodiversity conservation). These interfaces represent complex multi-host and multi-pathogen systems that have been so far little studied (13). Even if focusing on a single pathogen, the large diversity of hosts constrains the efficiency of past and current surveillance and control approaches. New frameworks are therefore needed bridging biological fields (14, 15). As pathogens have a limited number of transmission modes to infect a new host (e.g. direct, environmental such as water-borne, foodborne or

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vector/insect-borne transmission), a framework trying to identify the transmission processes linking one host to different sources of pathogens could help identifying hotspots of pathogen transmission and predicting future microorganism transmission at a local level (16, 17).

The bacteria *Escherichia coli* is a good indicator of transmission pathways within multi-host systems because *E. coli* is ubiquitous, shares the same niche as enteric pathogens and transferred by the same route and one of the best-studied and known bacteria. *E. coli* diversity and population dynamics have been the focus of recent studies (17-20) investigating the relationship between *E. coli* populations and proxies of inter-host contacts. For example, *E. coli* sharing between human, primates and livestock increased with the frequency and intensity of inter-species contacts in Uganda (21). However, more studies are needed with different animal models, in different ecosystems and using the new available molecular tools to characterize bacterial diversity. The dissemination of ABR in pristine ecosystems can also be used to track directional genetic transfer from human and livestock towards wildlife (10, 22, 23).

A wealth of studies exists on the host, temporal stability and geographical structure of *E. coli* associated with humans and domestic animals (e.g.(24-26). The factors contributing to the sharing of *E. coli* between host populations are: 1) feeding modes; 2) phylogenetic relatedness and 3) host contact patterns related to bacterial transmission (27). As it can be difficult to weigh each factor against each other, estimating the proportion of *E. coli* population similarity related to the last factor could be difficult. However, a recent study (28) provided a semi-experimental set-up that we used here. The animal model offers a good opportunity to investigate *E. coli* population sharing between hosts as the African buffalo (*Syncerus caffer caffer*) and cattle (*Bos taurus/indicus*) are bovids and therefore phylogenetically related, their diets overlap substantially and telemetry studies indicate that both populations can come into contact (28). Finally, ungulate population movements can be

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used to detect the degree of contacts between populations defining a contact variable that can be used to test hypotheses on *E. coli* population sharing.

The study was therefore initiated with a double objective: first to increase the knowledge on the dissemination of ABR genes between hosts in these complex systems, so far little studied, in order to assess the risk associated with this anthropological threat on natural ecosystems; secondly, to explore the processes of *E. coli* transmission between hosts as a model for pathogen transmission and potentially as a predictive tool. Hence, the genetic structure of commensal *E. coli* population and their ABR were explored simultaneously in sympatric ungulate hosts. We hypothesized that 1) the phylogenetic proximity and the diet overlap between cattle and buffalo in our study site would result in similar *E. coli* phylogroups' profiles but that 2) ABR in buffalos should increase with the level of habitat sharing with domestic hosts, as the use of antibiotics is restricted to human and domestic populations.

MATERIALS AND METHODS

Study site and experimental setting. The study was conducted in the Hwange district of Zimbabwe, Africa. The Hwange National Park (HNP) and its periphery (including the Sikumi Forest and surrounding communal lands) are part of the Kavango-Zambezi Transfrontier Conservation Area (KAZA TFCA) (28) (Figure 1). In southern Africa, TFCAs aim at combining sustainable development and biodiversity conservation through the promotion of the sustainable use of natural resources and agricultural production (29). The livelihoods of small-scale farmers rely heavily on basic livestock production (herd average n=5), little or no agricultural input (fertilizer, antibiotic feeds) (28) and maize and sorghum cropping in a semi-arid ecosystem (average rainfall 600mm per year).

The telemetry protocol presented in (28) targeting sympatric buffalo and cattle

populations was carried out on the same ungulate populations as of December 2012. Adult females were equipped with GPS collars as their movements are representative of the herd movements (30, 31). Annual home ranges for each individual/herd were calculated using the 95% Utilization Distribution method (32) and are displayed in Figure 1. *E. coli* sampling protocols were implemented in 3 populations identified using the telemetry results: a distant buffalo population (A) whose home range does not overlap with the other two populations (population size estimated around 1000 individuals); a neighboring buffalo population (B) (population size estimated also around 500 individuals) and a cattle population (C) (several hundred individuals) sharing Sikumi Forest.

In this area, interviews with animal health technicians, farmers and human health professionals revealed that antibiotics were used in cattle populations to treat tick-borne diseases and other infections (they were asked to list by order of importance the antibiotic they use or prescribe). The antibiotics used most frequently in cattle were mainly tetracycline, followed by oxytetracycline, penicillin and streptomycin (principally injected intra-muscularly). There does not appear to be any preventive use of antibiotics in the area in cattle. In human, antibiotics were mainly used to treat human tuberculosis (*Mycobacterium tuberculosis*), an infection with a high prevalence in the area (especially due to the high HIV burden). The main antibiotics used in humans were trimethoprim, cotrimoxazole (a combination of trimethoprim and sulphonamids), amoxicillin and doxycycline.

Sample collection. Fresh fecal samples of animals from the three populations A, B and C were collected on the ground a few seconds or minutes after deposition between October 31st and November 04th 2012. For cattle, the protocol was implemented in two villages (i.e. Magoli and Jwapi), following cattle herds returning from their daily roaming in the Sikumi forest to the kraal (i.e. overnight enclosure located close to homestead) before sunset. For buffalo populations (A and B), the herd was located using recent GPS positions transmitted

by satellite and very high frequency devices. After visual contact was established with the buffalo herd, movements were monitored, and the samples were collected just after the herd moved out of an open area. This protocol ensured that the fecal material collected was obtained from the right host (population A, B or C) and endeavored to minimize the sampling of fecal material from the same individuals by selecting distant dungs (more than 10 meters) or dungs with clear dissimilarities in color and/or density. The sample size of around 50 (5% or 10%) was estimated from the population size (500 heads for each buffalo populations and several hundred heads for the cattle population) and practically to minimize double sampling of individuals and taking into account laboratory time and costs. Labeled with unique identifying numbers, transport swabs (Clinical Sciences Diagnostics containing Amies transport medium) were immersed in the fecal material and transported in a cool box with ice packs from the field to a deep freezer (in less than 6h) in the research camp where they were then maintained at -20°C. During the same week, they were transported by car to Harare, capital of Zimbabwe, without de-freezing and stored in another deep freezer until shipment by plane to the INSERM laboratory in France in March 2013. Once in the laboratory, each swab was then discharged in brain heart infusion (BHI) broth with 20% glycerol and stored at -80°C until used.

Isolation of the dominant *E. coli* clone. The stool-containing suspensions were plated onto Drigalski agar plates and incubated overnight at 37°C. Then, one yellow colony was randomly picked and confirmed by MALDI-TOF (MALDI Biotyper Microflex, Bruker) to belong to the *E. coli/Escherichia* clade species. This colony was considered to represent the dominant *E. coli/Escherichia* clade clone as it has been recently shown (33, 34). The strain was tested for antibiotic susceptibility, phylotyped, and stored at -80°C. The used nomenclature for the designation of these strains was as follow: the letter of the population, the number of the individual and "DOM" for dominant (e.g. $B24_{DOM}$).

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178 **Antibiotic resistance.** Two protocols were used to analyze ABR. First, global ABR was
179 analyzed in each sample by plating 100 µl of the glycerol dilution on Drigalski agar on which
180 antibiotics disks containing amoxicillin (25µg), kanamycin (30UI), streptomycin (10UI),
181 tetracycline (30UI), trimethoprim (5µg), sulphonamids (200µg) and chloramphenicol (30µg)
182 were plated, as described (35). Plates were incubated 24h at 37°C and if colonies were present
183 within the zone of inhibition (as defined by the French Society for Microbiology, www.sfm-
184 microbiologie.org/) the sample was reported to be carrying resistant *Enterobacteriaceae*. A
185 Murray score was calculated as in (36) by the following equation : Murray score = (total
186 number of resistance per total number of possible resistances for each individual sample). In
187 addition, one randomly selected yellow colony falling within the zone of inhibition of
188 tetracycline, amoxicillin and trimethoprim was purified on Mueller-Hinton medium with the
189 corresponding antibiotic disk each time it was present. The *E. coli/Escherichia* clade
190 identification was confirmed by MALDI-TOF and stored at -80°C. Those strains were then
191 called tetracycline, amoxicillin and trimethoprim resistant strains, respectively, and labeled by
192 the letter of the population, the number of the individual and the abbreviation of the antibiotic
193 (*e.g.* $B24_{TET}$).

194 Secondly, classical antibiotic susceptibilities were determined using the disk diffusion
195 method according to the 2012 recommendations of the French Society for Microbiology on
196 the dominant and on the tetracycline, amoxicillin and trimethoprim resistant (see above) *E.*
197 *coli* strains. The following antimicrobial agents were tested:amoxicillin (25µg), amoxicillin +
198 clavulanic acid (20+10µg), ticarcillin (75µg), cefoxitin (30µg), cefepime (30µg), cefotaxime
199 (30µg), ceftazidime (30µg), streptomycin (10UI), gentamicin (10UI), kanamycin (30UI),
200 tetracycline (30UI), trimethoprim (5µg), sulfonamids (200µg), chloramphenicol (30µg),
201 nalidixic acid (30µg) and ofloxacin (5µg).

202 Further characterization was performed on the subdominant antibiotic resistant strains.

Detection of tetracycline resistance efflux pumps - encoding genes [*tet(A)* to *tet(E)*] using a multiplex PCR (37) was performed on the tetracycline resistant *E. coli* strains. Beta-lactamase encoding gene bla_{TEM} was screened by PCR (38) followed by Sanger sequencing on the amoxicillin resistant E. coli strains. Multiplex PCR detection of dihydrofolate reductase encoding genes *dfrA1, dfrA5/14, dfrA7/17* and *dfrA12* was performed, followed by Sanger sequencing, on the trimethoprim resistant E. coli strains. The choice of these genes was based on their prevalence in the *E. coli* genome database Mage (http://www.genoscope.cns.fr/agc/microscope/home/)(39). The primers for the dfrA PCR and the length of the PCR products were as follow: dhfr1.f AACCAATGGCTGTTGGTTGG, dhfr1.r CTGAAACAATGACATGATCCG, 180bp; dhfr5.f CCACCAGACACTATAACGTG, dhfr5.r CATACCCTGGTCCGCGAAAG, 237 bp; dhfr7.f TCAGAAAATGGCGTAATCGG, dhfr7.r ACGTGAACAGTAGACAAATG, 332bp; dhfr12.f TGAGACAAGCTCGAATTCTG, dhfr12.r TGAACTCGGAATCAGTACGC, 430 bp. The PCR conditions were as in (40). The differentiation between dfrA5 and dfrA14 genes on one hand, and dfrA7 and dfrA17 genes on the other hand, was performed by sequencing.

***E. coli* phylogenetic grouping and strain relatedness.** Dominant and subdominant tetracycline, amoxicillin and trimethoprim resistant *E. coli* strains were assigned to one of the 7 main phylogenetic phylogroups (A, B1, B2, C, D, E, F) using the new Clermont quadruplex method (40) or to one of the five *Escherichia* clades (I to V) as in (40, 41). The subdominant tetracycline resistant *E. coli* strain relatedness was assessed by repetitive extragenic palindromic PCR (rep-PCR) using a DiversiLab strain typing system (bioMérieux) as in (42). Relatedness among the strains was also assessed by random amplification of polymorphic DNA (RAPD) using the 1254 primer (5'-CCGCAGCCAA-3') as in (43).

Statistical analyses. Using the R software (44), after checking for homogeneity of variance (no distribution was normally distributed), non parametric tests (Kruskal-Wallis,

Wilcoxon, Chi-square and Spearman rank correlation tests) were implemented to compare the ABR and the phylogroup population structure between the three host populations.

RESULTS

We collected samples from 53 isolated wild buffalo (population A), 52 from neighboring wild buffalo (B) and 50 samples were collected from domestic cattle (C).

Phylogenetic group distribution and antibioresistance of the dominant *E. coli*/*Escherichia* clade strain. *E. coli/Escherichia* clade dominant strains were detected in 152 out of 155 samples [n=52 for buffalo (A), n=50 for neighboring buffalo (B) and n=50 for cattle (C)]. For three samples, the dominant *Enterobacteriaceae* did not belong to the *Escherichia* genus (two *Klebsiella oxytoca* in the B population and one *Enterobacter cloacae* in the A population).

The three host populations had similar patterns of *E. coli* phylogenetic group distribution (A-B: Spearman p=0.96, A-C Spearman p= 0.81, B-C Spearman p= 0.77) (Figure 2). B1 was the main phylogroup detected in those three populations (36-54% of the detected dominant strains) followed by the phylogroup E (24-34%). D phylogroup strains were present at more than 10% in population A. A, B2 and C phylogroups were rarely detected (less than 6%). Three *Escherichia* clade I strains were isolated, all in buffalo (2 in A population and 1 in B population). No phylogroup F strain was observed.

ABR was found very rarely in the dominant strains, as only one B1-phylogroup *E. coli* from the buffalo population at the interface with cattle ($B24_{DOM}$) was resistant streptomycin, tetracycline and sulphonamides.

Global antibiotic resistance of fecal *Enterobacteriaceae*. To have an overview of ABR in *Enterobacteriaceae*, the 155 fecal samples were tested for antibiotic resistant *Enterobacteriaceae* by direct plating, gathering by this approach both dominant and

253 subdominant strains (Table 1**)**. A significant difference between the ABR patterns of the three
254 populations was observed (Kruskal-Wallis Test $p<0.01$) (Table 1). Buffalo with no contact
255 with cattle (population A, average Murray score = 0.146) presented a lower Murray score than
256 buffalo at the interface (population B, average Murray score = 0.258) (Wilcoxon test: $p<0.01$)
257 and cattle (population C, average Murray score = 0.340) (Wilcoxon test: $p<0.01$). Cattle did
258 not exhibit a significantly higher resistant score than buffalo at the interface (B) (Wilcoxon
259 test: $p=0.21$). Trends by antibiotics were quite consistent: for 4 antibiotics out of 7
260 (tetracycline, trimethoprim, sulphonamid, chloramphenicol), we observed an increasing
261 antibiotic resistance along the gradient A<B<C; for 2 out 7 (streptomycin, amoxicillin), we
262 observed A<<C<B and for the remaining one, kanamycin, A=B<C. Specifically, tetracycline
263 resistance was significantly different between A and C (Chi-square test; $p<0.01$), A and B
264 (Chi-square test; $p=0.04$) and between B and C (Chi-square test; $p<0.05$). Amoxicillin
265 resistance was significantly different between A and C (Chi-square test; $p<0.01$), A and B
266 (Chi-square test; $p<0.01$) and between B and C (Chi-square test; $p<0.01$). Trimethoprim
267 resistance was significantly different between A and C (Chi-square test; $p<0.01$), not
268 significant between A and B (Chi-square test; $p=0.61$) and between B and C (Chi-square test;
269 $p<0.01$). In addition, buffalo (A and B, Murray score=0.201) had significantly less ABR than
270 cattle (C) (Wilcoxon test: $p<0.01$) and populations in contact (B and C, Murray score=0.298)
271 had significantly more resistance than isolated population (A) (Wilcoxon test: $p<0.01$).

272 ***E. coli* subdominant antibiotic resistant strains.** Due to the veterinary and human
273 medicine practices in Zimbabwe, we characterized further the presence of *E. coli*
274 subdominant strains resistant to tetracycline, which was the most commonly used antibiotic in
275 cattle, as well as resistance to amoxicillin and trimethoprim, which were largely used in
276 human. Furthermore, a very contrasted pattern of tetracycline resistant *Enterobacteriaceae*,
277 and at a lesser extend of amoxicillin and trimethoprim resistance, among host populations

were observed (Table 1). No antibiotic resistant *E. coli* strain was identified in the population A (buffalo with no contact with cattle) whereas 3 and 19 fecal samples yielded resistant *E. coli* strains in populations B (buffalo in contact with cattle) and C (cattle), respectively (significant difference between C and the two other populations; Chi-square test; $p<0.01$ for C and A; C and B) (Table 2). Using our strategy, we sometimes isolated several strains that were resistant to two or three of the tested antibiotics in a single sample. We considered that the strains were identical when they belonged to the same phylogroup, exhibited the same pattern of antibiotic resistance on the antibiogram, possessed the same resistance gene and shared an identical RAPD profile. Thus, 3 and 23 subdominant resistant strains were identified in populations B and C, respectively (significant difference between C and the two other populations; Chi-square test; $p<0.01$ for C and A; C and B) (Table 2). Of note, the subdominant resistant strain isolated in the B24 sample ($B24_{TET}$) was identical to the dominant strain resistant to antibiotics ($B24_{DOM}$), as confirmed by RAPD analysis.

In the isolated resistant strains, a high diversity of *E. coli* phylogenetic groups was observed with 5 phylogroups represented (Table 2). To document this heterogeneity further, we performed rep-PCR on the most frequently isolated subdominant tetracycline resistant strains (Figure 3). Only three B1 phylogroup strains from the cattle population ($C12_{TET}$, $C26_{TET}$, $C29_{TET}$) belong to the same clone. For the remaining strains, the rep-PCR did not reveal any identical strain between the buffalos at the interface and the cattle subdominant tetracycline resistant strain population. Similarly, B1 phylogroup strains $C2_{TMP}$, $C38_{TMP}$ and $C42_{TMP}$ gave all a RAPD clear distinct pattern. Of note, in the cattle population, the main phylogroups of the resistant subdominant strains were the A and B1 phylogroups (39.1% each) followed by the C phylogroup (13%), contrary to the B1 and E phylogroups for the dominant strains (Figure 2).

The antibioresistant strains were very rarely resistant to only one antibiotic (two strains

resistant only to tetracycline) but were resistant to up to 8 of the tested antibiotics. The most common ABR was the tetracycline and sulphonamid resistance (21 strains, 80.7% of (all) the resistant strains) followed by trimethoprim (18 strains, 69.2%), amoxicillin/ticarcillin (16 strains, 61.5%) and streptomycin (13 strains, 50.0%) resistances (Table 2). Six strains were resistant to the association amoxicillin/clavulanic acid and one to kanamycin. A multiplex PCR assay of *tetA* to *tetE* genes responsible of tetracycline resistance (37) identified a *tet* gene in all the tetracycline resistant strains (Table 2). The genes were mainly *tetA* and *tetB* and found in both buffalo and cattle populations. Only one cattle strain had *tetC*. A multiplex PCR assay of the *dfr* genes involved in trimethoprim resistance identified a majority of *dfrA14* genes but some *dfrA1, dfrA5, dfrA7* and *dfrA17* genes, the *dfrA5* and *dfrA7* genes being shared between buffalo and cattle populations. Lastly, we confirmed by PCR-sequencing that the amoxicillin resistance found in both populations was due to narrow-spectrum beta-lactamase TEM-1 (Table 2).

Altogether, these data indicate that diverse *E. coli* strains bearing antibioresistance genes (*tet, dfrA* and $bla_{TEM\text{-}1}$) are present in buffalo in contact with cattle and especially in cattle, but not in buffalo without contact with cattle.

Discussion

We explored the structure and the level of antibioresistance of *E. coli* commensal populations and the resistant *Enterobacteriaceae* carriage of sympatric buffalo and cattle populations characterized by their contact patterns in a southern African ecosystem. Our results 1) identify an ABR gradient that we genetically characterized from cattle to buffalo, structured by host phylogeny and contact patterns, 2) confirm our initial assumptions that buffalo and cattle shared similar phylogroup profiles, albeit with some variability that led us 3) to hypothesize the role of the human/animal interface in the diffusion of genetic material

328 from human to cattle and finally towards wildlife.

329 The main result of this study is the identification of an ABR gradient between sympatric
330 domestic and wild ungulate populations in a tropical ecosystem. We detected this gradient at
331 several levels. First, at the *Enterobacteriaceae* community level, the Murray score indicated
332 that the cattle population had significantly more ABR than buffalo and that ungulate
333 populations in contact (i.e. population B and C with overlapping home ranges) shared more
334 ABR than ungulate populations that were not in contact (population A) (Table 1, Figure 1).
335 Secondly, whereas almost no ABR was detected in dominant *E. coli* strains isolated from the
336 3 host populations, subdominant antibiotic resistant *E. coli* strains were mainly present in
337 cattle and, at a lower isolation ratio in the buffalo population in contact with the cattle
338 population, whereas antibiotic resistant *E. coli* strains were absent from the buffalo population
339 that had no contact with the 2 other populations (Table 2). Finally, the molecular
340 characterization of ABR associated with the observed various genetic backgrounds in the
341 subdominant resistant *E. coli* strains found in populations B and C suggested that these strains
342 rarely spread between individuals, contrary to the antibioresistance genes which are shared
343 within the cattle population as well as between buffalo and cattle at the interface. It can be
344 hypothesized that strains can be transmitted at the interface rapidly, but that antibioresistance
345 genes spread independently. This is facilitated by the fact that these genes are borne by
346 mobile genetic structures. In *E. coli, tet* efflux genes are found in transposons inserted into
347 diverse plasmids from a variety of incompatibility groups (45) and $bla_{\text{TEM-1}}$ has been observed
348 to disseminate on the Tn*3* transposon (46). Similarly, *dfr* genes are often integron-borne genes
349 (47). In those subdominant resistant strains, multiple resistance was observed (Table 2)
350 which is mainly conferred by mobile genetic elements. Such a mechanism of selfish gene
351 spread rather than strain or plasmid spread has recently been proposed to explain the
352 dissemination of acquired resistance to ß-lactams in small wild mammals in French Guiana

pristine forest from an Amerindian village (48).

ABR in natural ecosystems can originate from two sources: 1) natural ABR emerging in the wild through natural selection processes; or 2) diffusion of genetic material or organisms harboring these ABR from an anthropological origin, i.e. through the use of antibiotics in domestic animals or in humans and their subsequent diffusion in the environment (10). We are confident that the gradient identified originated from the latter process, because the main ABR detected in the buffalo population matched the most frequently used antibiotics in domestic animal and human populations (tetracycline and streptomtycin for domestic animal and trimethoprim and amoxicillin for humans); because ABR in cattle was also detected for antibiotics used in human populations; and because the resistance genes identified here have already been isolated in many different contexts, and their emergence is supposed to be a rare event. In addition, the buffalo population in contact with cattle had an intermediate degree of ABR both at the global and subdominant antibiotic resistant *E. coli* strains, and all ABR found in wildlife was also found in cattle. The dominant clone is usually the clone with the best fitness in a given environment. Many drug resistances confer a fitness cost (49), and it is likely that antibiotic resistant bacteria will be outcompeted in a low antibiotic pressure environment, such as protected areas. In this case, resistant clones will probably not be selected as dominant. However, several processes act to stabilize resistance (compensatory evolution) (50), and there is also evidence that the genetic adaptations to the costs of resistance can virtually preclude resistant *E. coli* lineages from reverting to sensitivity (51). This could explain that only one buffalo in contact with cattle has a dominant strain resistant to antibiotics (B24) (Table 2). Our data are in line with a worldwide study of commensal *E. coli* in wild and domestic animals that showed the anthropogenic origin of antibiotic resistance and integron, a molecular vector of resistance (8).

The profiles of *E. coli* populations between the three host populations shared a degree of

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similarity. The phylogenetic proximity of ungulate hosts and the fact that they seasonally use food and water resources in the same ecosystem (no supplementary feeding for cattle except for crop residues left in the fields in the study area) can explain these results. The dominant phylogroup for the three ungulate populations was B1, followed by phylogroup E (Figure 2), in agreement with available knowledge for ruminant populations (52). However, the third phylogroup prevalence differed between populations A, B and C. In cattle, the third most prevalent phylogroup was A, a dominant phylogroup for human populations (33), suggesting a transfer of strains between human and cattle that interact through frequent and close contacts (8). In buffalo, the third most prevalent phylogroups were D and B2 respectively for populations A and B, indicating that different subdominant phylogroup dominate in different populations of the same species, as suggested for humans (52). Interestingly, the subdominant resistant strains of the cattle population were mainly of phylogroup A (9/26) with only two strains of phylogroup E, as opposed to the dominant clones, suggesting also a transfer of human origin (52). However, this result was not observed in the buffalo populations, from which only 3 strains were isolated (Table 2).

Although the mechanisms of genetic material transfer are not known, we demonstrate that the level of ABR varies according to the contact patterns between host populations. Sharing pasture and water points offers opportunities for direct and indirect transfer of organisms or genetic materials between wild and domestic ungulates. Close contacts between human and cattle occur regularly, especially when cattle are kept in the kraal every evening, where lactating female are milked, and the herders manipulate animals. Often, human and livestock share a unique water source. These behaviors can explain the presence of phylogroups of potential human origin (i.e. phylogroup A) and ABR against human antibiotics. It has been recently shown in the Amazonian forest that acquired ABR did not disseminate in the wild far (600 meters) from the point of selective pressure represented by

the village (48).

These results are important at two levels. First, they provide some information on the dissemination of bacteria and their resistance at wild/domestic/human interfaces, indicating that wildlife populations within protected conservation area are not exempt from anthropological pollution, even in the most remote areas. The impacts of *E. coli* (and potentially other bacteria) and resistance genes transfers to wild populations are difficult to assess, but they could alter the microbiome structures in wildlife and affect their behavior and/or health (53, 54). This dissemination can also pose a threat to the domestic and human populations from which they originate, as resistance genes in different selective environments can evolve into more harmful variants when they are introduced back into domestic or human populations (55). Follow-up studies on the mechanisms of bacteria and gene diffusion in this ecosystem could be targeted at describing the human *E. coli* population structure and ABR and the role of other domestic and wild hosts and the environment.

Second, these results support the potential use of *E. coli* as an indicator of transmission pathways in multi-host systems as recently suggested (17). Dominant strains are shared between hosts in contact (e.g. phylogroup B1 and E between cattle and buffalo; potentially A between human and cattle) and offer a first level of variability to be used to assess transmission processes between hosts. If resistant sub-dominant strains were not shared between in-contact host populations, their ABR genes were, identifying a second level of exploitable variability and a directional transmission pathway from cattle to buffalo, with humans as the probable source population. The intensity, frequency and directionality of these transmission events between hosts could be further investigated using new next generation sequencing tools targeting specific genetic sequences and applied to time series of multi-host sampling coupled with studies estimating proxies of inter-host contacts. For example, Miguel et al. (28) indicated seasonal and inter-annual inter-host contact patterns that could translate

into pulses of ABR dissemination. The outcome would be a framework to identify "highways" of transmission between hosts, with potential spatial and temporal variability, giving a head-start to the surveillance of emerging disease spillover events.

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441 **Table 1**: Global antibiotic resistance prevalence of fecal *Enterobacteriaceae* for each
442 ungulate population

	Host population A[1] n=53	Host population B n=52	Host population C n=50
Antibiotic			
Streptomycin	2 (3.8)[2]	9 (17.3)	8 (16.0)
Tetracycline	0	4 (7.7)	17 (34.0)
Amoxicillin	20 (37.7)	45 (86.5)	34 (68.0)
Trimethoprim	9 (17.0)	11 (21.2)	23 (46.0)
Sulphonamid	20 (37.7)	20 (38.5)	25 (50.0)
Kanamycin	2 (3.8)	2 (3.8)	5 (10.0)
Chloramphenicol	1 (1.9)	3 (5.8)	7 (14.0)
Murray resistance score[3] (mean and confidence interval)	0.146 (± 0.150)	0.258 (± 0.204)	0.340 (± 0.275)

443 [1] A = Buffalo population not in contact; B = buffalo population in contact with cattle; C =
444 cattle population.

445 [2] For each antibiotic, the first number represents the number of resistant samples and the
446 second, the related percentage for the given host population.

447 [3] The Murray score was calculated for all antibiotics as in Murray et al. (36).

448 **Table 2**: Antibiotic resistant phenotype and phylogenetic group belonging of each antibiotic resistant subdominant *E. coli* strain

449 detected in two out of three host populations.

450

Isolate ID[1]	Antibiotic-resistant phenotype[2]	*tet* gene	*bla*$_{TEM-1}$ *gene*	*dfr gene*	*E. coli* phylogenetic group[3]
Buffalo at the interface (B)					
$B1_{TET}$	TET, SMN, AMX, TMP, SUL, TIC	A	+	*dfrA5*	C
$B4_{TET}$	TET, SMN, AMX, TMP, SUL, TIC	A	+	*dfrA7*	D
$B24_{TET}$[4]	TET, SMN, SUL	B	ND[5]	ND	E
Cattle (C)					
$C1_{TET}$	TET	A	ND	ND	A
$C2_{TMP}$	TMP, SUL	ND	ND	*dfrA14*	B1
$C9_{TET}$	TET, SMN, AMX, TMP, SUL, TIC	A	+	*dfrA14*	A
$C12_{TET}$	TET, SMN, AMX, TIC	B	+	ND	B1
$C18_{TET}$	TET	A	ND	ND	A
$C18_{AMX}$	TET, AMX, SUL, TIC, AMC	A	+	ND	C
$C25_{TET}$	TET, AMX, TMP, SUL, TIC, AMC	A	+	*dfrA14*	B1
$C26_{TET}$	TET, SMN, AMX, TIC	B	+	ND	B1
$C26_{TMP}$	TMP, SUL	ND	ND	*dfrA14*	A
$C29_{TET}$	TET, SMN, AMX, TIC	B	+	ND	B1
$C31_{TMP}$	TET, TMP, SUL	B	ND	*dfrA14*	A
$C32_{TET}$	TET, SMN, AMX, TMP, SUL, K, TIC, AMC	B	+	*dfrA1*	C
$C36_{TET}$	TET, SMN, AMX, TMP, SUL, TIC	A	+	*dfrA1*	A
$C36_{TMP}$	TET, AMX, TMP, SUL, TIC, AMC	A	+	*dfrA14*	C
$C37_{TET}$	TET, SMN, AMX, TMP, SUL, TIC	A	+	*dfrA7*	A
$C38_{TMP}$	TMP, SUL	ND	ND	*dfrA14*	B1
$C40_{TET}$	TET, AMX, TMP, SUL, TIC, AMC	A	+	*dfrA14*	A
$C42_{TMP}$	TMP, SUL	ND	ND	*dfrA14*	B1
$C43_{TET}$	TET, TMP, SUL	B	ND	*dfrA17*	E

$C43_{TMP}$	TET, SMN, AMX, TMP, SUL, TIC	A	+	*dfrA5*	B1
$C44_{TET}$	TET, TMP, SUL	A	ND	*dfrA17*	A
$C45_{TMP}$	TMP, SMN, AMX, SUL, TIC	ND	+	*dfrA7*	B1
$C46_{TET}$	TET, SMN, AMX, TMP, SUL, GM, TIC, AMC	C	+	*dfrA17*	D

[1] The strains are labeled by the letter of the population, the number of the individual and the abbreviation of the antibiotic on which they were isolated. When a strain was isolated on several antibiotics, only one is arbitrarily presented.
[2] Abbreviations are as follow: amoxicillin (AMX), amoxicillin + acid clavulanic (AMC), ticarcillin (TIC), streptomycin (SMN), gentamicin (GM), kanamycin (K), tetracycline (TET), trimethoprim (TMP) and sulphonamid (SUL).
[3] Determined as in (Clermont et al. 2013).
[4] This strain was identical to the dominant strain ($B24_{DOM}$) as shown by RAPD.
[5] ND: not determined.

Figure 1: Study site including home ranges (95% UD, 2012-2014) of adult female buffaloes were drawn in red and cattle drawn in green. Herd A (4 GPS collars, approx. 1000 individuals) roamed in Hwange NP (dark grey) and herd B (4 GPS collars, approx. 500 individuals) remained in Sikumi forest (grey) and privately owned safari areas (light gray). Three cattle homeranges drawn in green (95% UD, 2010-2011) were representative of cattle living in Magoli and Jwapi villages in Hwange Communal Area (white) and entering Sikumi Forest. No fence separates any of the landuse displayed.

Figure 2: *E. coli/Escherichia* clade phylogenetic distribution of the dominant clones for each of the three ungulate populations: Population A (buffalo not in contact, n=53,), Population B (buffalo at the interface, n=52), Population C (cattle, n=50). Phylogroups A, B1, B2, C, D, E and *Escherichia* clade I (Clade) are displayed for each host population (no phylogroup F was observed).

Figure 3: Comparison of *E. coli* subdominant tetracycline resistant strains by repetitive extragenic palindromic PCR using a DiversiLab strain typing system (bioMérieux, Marcy l'Etoile, France). The Clermont genotypes determined as in Clermont et al. (2013) are indicated on the right of the figure.

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