Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Antimicrobial resistance in faecal samples from buffalo, wildebeest and zebra grazing together with and without cattle in Tanzania

A.A.S. Katakweba[1], K.S. Møller[2], J. Muumba[3], A.P. Muhairwa[4], P. Damborg[2], J.T. Rosenkrantz[2], U.M. Minga[5], M.M.A. Mtambo[4] and J.E. Olsen[2]

1 Pest Management Centre, Sokoine University of Agriculture, Morogoro, Tanzania
2 Department of Veterinary Disease Biology, University of Copenhagen, Frederiksberg C, Denmark
3 Ngorongoro Conservation Area Authority, Ngorongoro Crater, Arusha, Tanzania
4 Department of Veterinary Medicine and Public Health, Sokoine University of Agriculture, Morogoro, Tanzania
5 Faculty of Science, Technology and Environmental Studies, The Open University of Tanzania, Dar Es Salaam, Tanzania

Keywords
antibiotic resistance, buffalo, cattle, Tanzania, wildebeest, zebra.

Correspondence
John Elmerdahl Olsen, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbøjlen 4, DK-1870 Frederiksberg C., Denmark.
E-mail: jeo@sund.ku.dk

2014/1429: received 13 July 2014, revised 28 November 2014 and accepted 19 December 2014

doi:10.1111/jam.12738

Abstract

Aim: The aim of this study was to determine whether the practice of co-grazing with cattle and wild life constitutes a risk of transmission of antibiotic resistant bacteria to wild ungulates.
Methods and Results: Faecal samples were collected from buffalo (n = 35), wildebeest (n = 40), zebra (n = 40) and cattle (N = 20) from Mikumi National Park, Tanzania (MNP), where cattle is prohibited and from Ngorongoro Conservation Area (NCA) where co-grazing is practiced. The number of coliforms and enterococci resistant to selected antibiotics was determined. Wild life generally harboured higher number of resistant *Escherichia coli* and Enterococci than cattle, but with no general influence in wild life of co-grazing with cattle. Vancomycin-resistant Enterococci were detected in wild life samples, and *E. coli* resistant to cefotaxime and enrofloxacin were observed among isolates from all wild life, but not from cattle. Culture independent estimates of the number of *sulII* gene copies obtained by qPCR did not differ between wild life from the two sample sites, while *tetW* was significantly higher in samples from MPN than from NCA.
Conclusions: Antibiotic resistant bacteria were not more frequently found in ungulates grazing together with cattle than ungulates without this interaction.
Significance and Impact of the Study: This study did not indicate that transmission of antibiotic resistant bacteria is a frequent event following co-grazing of wild life and cattle.

Introduction

Wild life animals often carry antibiotic resistant bacteria. For example, healthy wild scavenging birds such as seagulls (Radhouani *et al.* 2009) and birds of prey such as buzzards (Radhouani *et al.* 2012) have been found to carry high numbers of antibiotic resistant bacteria in the absence of any known contact with antibiotics. Such bacteria are believed to originate in humans and domesticated animals (Skurnik *et al.* 2006), and they form an indirect proof of transmission of either the resistant bacteria or horizontal transfer of resistance genes. Increased human activities within the habitats of wild animals increases the risk of interspecies disease transmission, and areas of high human density or intense research and ecotourism activities expose wildlife to a high risk of disease spillover from humans and livestock (Benavides *et al.* 2012).

Tanzania has a high number of wildlife animals, and wild life tourism constitutes an important part of the economy. The animals are located in 15 national parks as well as conservation areas, such as the famous Ngorongoro

Conservation Area (NCA). The latter is Tanzania's most visited protected area, and in addition it is a multiple land-use zone inhabited not only by the wild life but also by the pastoral Massai people keeping Tanzania Short Horned Zebu cattle in high numbers. Within NCA the main wild ungulates, wildebeest (*Connochaetes taurinus*), zebra (*Equus burchelli*) and buffalo (*Syncerus caffer*), interact with Massai people and their cattle in the grazing land and at water points (Voeten and Prins 1999; Charnley 2005). While antibiotic resistance has been described in African wild primates (Rolland *et al.* 1985), mountain gorillas (Rwego *et al.* 2008), wild boars (Literak *et al.* 2009) and mongoose (Pesapane *et al.* 2013), there is limited information available on the levels of antibiotic resistance from wild ungulates, and in particular how this is influenced by sharing grazing land with domesticated animals and the general environment with the pastoral people.

Escherichia coli and *Enterococcus* spp. are normal inhabitants of the gastrointestinal tracts of humans and most animal species (Lester *et al.* 2006), and they are good indicators of local selection pressures (Aarestrup 1999; Byarugaba *et al.* 2011). The aim of this study was to determine whether co-grazing with cattle influences antimicrobial resistance in *E. coli* and *Enterococcus* spp. from faecal samples of wild ungulates. This was assessed by comparing resistance levels in faecal samples from three wild animal species from NCA with interaction with cattle with levels in faecal samples in the same species of animals from the Mikumi National Park (MNP), where cattle are strictly prohibited to graze, and where wild animals have little interaction with humans.

Materials and methods

Research permit

Research permits needed to perform this study was obtained from Tanzania Commission for Science and Technology (COSTECH) (permit No. 2010-324-NA-2010-161 dated 1st November 2010) to carry out research in NCA and MNP in collaboration with Tanzania Wildlife Research Institute (TAWIRI).

Study area

The study was carried out in NCA, Tanzania with GPS-coordinates 3°12′S 35°27′E/3·209°S 35·46 (UNESCO, 2011) and in MNP with GPS-coordinates 7°12′S 37°08′E/7·200°S 37·13 (Fig. 1) between November 2010 and October 2011. The NCA has a population of about 25 000 large animals (Berry 2009), including the three species investigated in the current study: wildebeest, zebra and buffalo. Masai people graze cattle in high numbers within this area; a 2013 census estimated 136 500 cattle and 292 000 small ruminants. MNP is the fourth-largest park in Tanzania, and part of a much larger ecosystem centred on the uniquely vast Selous Game Reserve (TANAPA, 2013). It has a wild life population of large animals of *c.* 15 000 heads (Marttila 2011), including the three species investigated in the current study. Cattle are prohibited within the borders of this park.

Sample sources and sampling

One freshly voided faecal sample (3–5 g) was collected from each buffalo (NCA $n = 15$: MNP $n = 20$), wildebeest (NCA $n = 20$; MNP $n = 20$), zebra (NCA $n = 20$; MNP $n = 20$) and zebu cattle (NCA $n = 20$). All samples in NCA were collected on 1 day in the month of November 2010 and all samples in MNP on 1 day October 2011. One-day sampling was chosen to avoid sampling the same animal more than one time. Wild life and cattle were tracked on foot until defecation was observed. Samples from buffalo, zebra and cattle, which defecate voluminous, were collected from the inside of the dung using plastic gloves. Dung from wildebeest is small, and here the majority of the dung was used, however, taking care not to include the part that had touched ground. After collection, samples were stored in sterile containers in the cooling box with ice at *c.* 4°C and transported to a local laboratory for analysis on the day of collection.

Enumeration of antibiotic resistant indicator bacteria

Tenfold serial dilutions of faecal material were made in phosphate buffer saline (PBS). For enumeration of total and resistant coliforms, 100 μl of the suspensions were spread on MacConkey agar (Oxoid, Basingstoke, UK) plates without antibiotics and plates containing different antibiotics: 2 mg l^{-1} cefotaxime (CTX), 16 mg l^{-1} tetracycline (TE), 16 mg l^{-1} ampicillin (AMP), and 256 mg l^{-1} sulfamethizole (SUL). Enumeration of total and resistant enterococci was carried out on Slanetz-Bartley agar plates (Oxoid) without antibiotics, and on plates containing either 6 mg l^{-1} AMP or 16 mg l^{-1} vancomycin (VA). Concentrations of antibiotics were chosen based on MIC distributions for *E. coli* and *Enterococcus faecium* shown in EUCAST (EUCAST 2011) to obtain a good separation between sensitive and resistant populations. MacConkey plates were incubated for 1 day and Slanetz-Bartley plates for 2 days at 37°C. Counts were estimated by weighted average from plates with between 10 and 200 colonies. In statistical comparisons between groups of animals, animals with counts below the

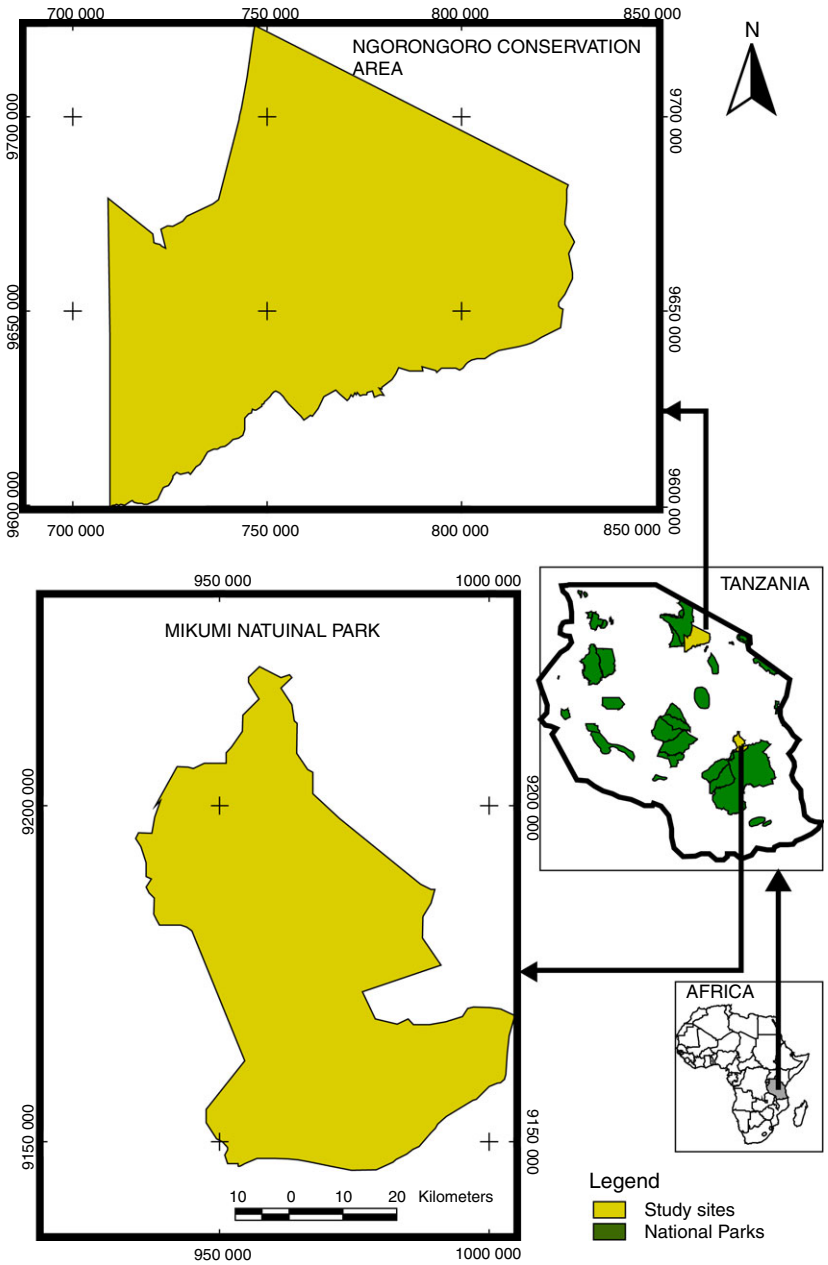

Figure 1 Overview of location of the two sampling sites, Ngorongoro Conservation area and Mikumi National Park in Tanzania. (▭) Study sites and (■) National Park.

detection level of 100 CFU g^{-1} were given a value of 1 CFU g^{-1}.

Bacterial species identification

One or two colonies of *E. coli* and enterococci with typical colony morphology from each plate were sub-cultured in order to get pure cultures. These cultures were stored at −80°C until further analysis. Species identification of lactose positive colonies from MacConkey agar was performed using Gram stain and the IMViC phenotypic tests (indole, methyl red, Voges Proskauer and citrate) using standard procedures. Presumptive *Enterococcus* colonies were species identified by multiplex PCR according to Dutka-Malen *et al.* (1995). Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Vitek MS RUO, bioMérieux, Marcy l'Etoile, France) was used to identify isolates that did not show typical reactions in the biochemical tests or by multiplex PCR.

Antibiotic sensitivity testing

Randomly picked and purified isolates that had been characterized to species level and originating from MacConkey

and Slanetz-Bartley plates without antibiotics were subjected to antimicrobial susceptibility testing by the disc diffusion method according to CLSI (2008) guidelines. For *E. coli,* the following discs were used: sulphamethoxazole/trimethoprim 19 : 1 (SXT 25 μg), ampicillin (AMP 10 μg), amoxicillin-clavulanic acid 2 : 1 (AMC 30 μg), gentamicin (CN 10 μg), tetracycline (TE 30 μg), enrofloxacin (Ent 10 μg) and cefotaxime (CTX 30 μg). For *Enterococcus* spp., antimicrobials were sulphamethoxazole/trimethoprim 19 : 1 (STX 25 μg), erythromycin (E 15 μg), ampicillin (AMP 10 μg), rifampicin (RD 5 μg), gentamicin (CN 120 μg) and tetracycline (TE 30 μg). The reference strains *E. coli* ATCC 25922 and *Ent. faecium* ATCC 2912 were used for quality control. Colonies showing resistance to CTX were analysed for ESBL properties using the synergy test (Lewis *et al.* 2007).

Quantitative PCR

After thorough vortex, 200 μl of the 10^{-1} dilution used for quantification of resistant bacteria by colony counts above, were used for DNA extraction with QIA amp DNA Stool Mini Kit (Cat 51504; Qiagen, Copenhagen, Denmark) following manufacturer's instructions. DNA was stored at −20°C until use.

Quantitative PCR (qPCR) for enumeration of two antibiotic resistance genes and for culture independent estimation of the population number was performed using previously designed primers for *sulII* (*sulII*-Fw: TCCGGTGGAGGCCGGTATCTGG; *sulII*-Rv: CGGGAATGCCATCTGCCTTGAG), *tetW* (*tet(W)*-Fw: GAGAGCCTGCTATATGCCAGC; *tet(W)*-Rv: GGGCGTATCCACAATGTTAAC) and 16s rRNA (1369-Fw: CGGTGAATACGTTCYCGG; 1492-Rv: GGWTACCTTGTTACGACTT) (Suzuki *et al.* 2000; Aminov *et al.* 2001; Pei *et al.* 2006). The qPCR reactions were performed using either a Stratagene MX 3000p PCR cycler with 25 μl reactions (12·5 μl Maxima SYBR Green/ROX qPCR Master mix (Fermentas, Thermo Scientific, Copenhagen, Denmark); 0·3 μmol l^{-1} of each primer and 5 μl template) or a Roche LightCycler 96 PCR cycler with 25 μl reactions (12·5 μl FastStart Essential DNA Green Master (Roche, Hvidovre, Denmark); 0·3 μmol l^{-1} of each primer and 5 μl template). The programme consisted of an initial 10 min at 95°C followed by 40 cycles of 15 s at 95°C; 30 s at the annealing temperature (57°C for *sulII*, 60°C for *tetW* and 16s rDNA); 30 s at 72°C. At the end of the programme a dissociation curve stage was included. Standard curves were used for calculation of the gene copy number per gram dung sample, constructed by running samples of known gene copy number calculated using the concentration of the template, measured using NanoDrop (Thermo Scientific), and the fragment length. Representative curves are shown as Figure S1–S3. The prediction interval of the sample content was calculated using the formulas developed by Danzer *et al.* (1998).

Statistical analysis

Statistical Package for Social Science software (SPSS) ver. 16.0 packages for Windows® was used for analysis of susceptibility data by the chi-square test. The test for independence, with degree of freedom, where applicable, was used to compare the prevalence of resistance to antimicrobial agents among isolates from different sources. SAS ver. 9.1 (SAS, 2011) was used to analyse overall variation in bacterial counts and number of genes from qPCR using ANOVA with Duncan's postmodification test.

Results

Counts of total and resistant coliforms and *Enterococcus* spp.

As shown in Table 1, the coliform counts were often below the detection level of 100 CFU g^{-1}, most commonly in cattle samples. Overall, levels of antibiotic resistant coliforms did not differ significantly between the three wild life species. Although mostly nonsignificant, counts of resistant coliforms were generally lower in cattle than in wild life, however, the proportions of total coliforms that were made up of resistant coliforms (resistant coliform divided by total coliform counts) were very

Table 1 Quantification (Log_{10} CFU ± SE g^{-1}) of resistant *Escherichia coli* from wild life species and cattle

Antibiotics/species	Ampicillin	Tetracycline	Cefotaxime	Sulphamethoxazole/trimethoprim	No antibiotic
Buffalo	2·42 ± 0·33^{a} (22/35)	1·56 ± 0·28^{a} (17/35)	1·00 ± 0·25^{a} (9/35)	1·99 ± 0·3^{a} (21/35)	4·84 ± 0·25^{a} (35/35)
Zebra	2·04 ± 0·33^{a} (21/40)	1·15 ± 0·28^{a} (13/40)	0·39 ± 0·25^{a} (5/40)	1·43 ± 0·3^{ba} (15/40)	4·25 ± 0·24^{a} (40/40)
Wildebeest	1·91 ± 0·33^{a} (20/40)	0·78 ± 0.28^{a} (9/40)	0·93 ± 0·25^{a} (8/40)	1·27 ± 0·3^{ba} (14/40)	4·60 ± 0·25^{a} (40/40)
Cattle	1·95 ± 0·52^{a} (8/20)	0·68 ± 0·44^{a} (4/20)	0·29 ± 0·4^{a} (2/20)	0·62 ± 0·48^{b} (3/20)	3·14 ± 0·39^{b} (20/20)

Groups with the same superscript are not statistically different. Number in brackets shows how many samples out of tested samples that had CFU above the detection level of 100 CFU g^{-1}. Samples below this level were assigned a value of 1 CFU g^{-1} for statistical purposes.

similar in wild life and cattle (Table S1). Comparing data from wild life in the two parks, counts of AMP-resistant coliforms were significantly higher in wild life samples from NCA compared to wild life samples from MNP. Counts of CTX-resistant coliforms were significantly higher in samples from MNP. One or two colonies were isolated from all samples ($N = 24$) that showed growth on the CTX plates. All resistant isolates showed synergy with clavulanic acid in ESBL test, indicating presumptive ESBL isolates.

There was no significant difference in tetracycline resistance in wild life between the two study sites, while counts of sulphamethoxazole-resistant bacteria were higher in samples from MNP. The average total number of coliforms in wild life from MNP was significantly higher than the number of coliforms in wild life from NCA.

Vancomycin-resistant (VRE) and AMP-resistant *Enterococcus* were not observed in samples from cattle, while a total of 10 wild life animals carried VRE and AMP-resistant enterococci (Table 2). The VRE were found in one buffalo, eight zebras and one wildebeest, and was shown to belong to *Ent. faecium, Enterococcus faecalis* and *Enterococcus hirae*. Though on average the level of resistance to these antibiotics was low in enterococci, the counts of AMP-resistant *Enterococcus* spp. were found to be significantly higher ($P < 0{\cdot}05$) in samples from MNP compared to NCA.

Species composition of bacteria obtained from MacConkey and Slanetz-Bartley agar

The value of indicator bacteria relies heavily on the assumption that bacteria compared from different animals belong to the same species. To ensure that this was the case before isolated colonies were characterized by disc diffusion assay, 120 presumptive *E. coli* and 120 presumptive *Enterococcus* spp. were randomly selected and identified to the species level. All lactose positive colonies selected from MacConkey agar were demonstrated to be *E. coli*. The species distribution of the *Enterococcus* isolates is shown in Table 3. Of the 120 isolates, 95 (79·2%) belonged to *Ent. faecium*, and the rest included *Ent. faecalis* (7·5%), *Enterococcus gallinarum* (5·8%) and *Ent. hirae* (7·5%). *Ent. gallinarum* was found in Wildebeest and Zebra, and *Ent. hirae* was found in all wild ungulates, but not in cattle. No significant difference in species composition was observed between animals from NCA and MNP (data not shown).

Antibiotic susceptibility of *Escherichia coli* and *Enterococcus* spp.

Wild life isolates of *E. coli* from MNP were more frequently resistant to tetracycline ($P = 0{\cdot}03$), gentamicin ($P < 0{\cdot}001$) and enrofloxacin ($P < 0{\cdot}0001$) than isolates from NCA (Table 4). Significant differences were also observed between animal species, for example AMP resistance was significantly more common in isolates obtained from buffalo than the other species investigated. Notably, *E. coli* resistant to CTX and enrofloxacin were observed among isolates from all wild life, but not from cattle. Colonies showing resistance to CTX all showed synergy with clavulanic acid, indicating that a gene of the ESBL-group encoded the resistance.

Enterococci from wild life animals in NCA and MNP only differed with respect to sulpha-trimethoprim resistance with isolates from NCA showing the highest prevalence (Table 5). Contrary to *E. coli* isolates, there was no significant difference in resistance level between animal species.

Quantification of resistance genes *tetW* and *sulII*

To investigate whether differences observed above were a reflection of inherent overall differences in resistance levels in the total intestinal flora, two antibiotic resistance genes were selected and quantified by qPCR, i.e. a culture independent approach. The copy number of *sulII* genes did not differ in wild life between the two locations and did not differ between animal species, whether this was expressed as absolute numbers or as a proportion of 16s rRNA copy number (Table 6). In contrast, animals from NCA had a higher number of *tetW* genes

Table 2 Quantification (Log_{10} CFU ± SE g^{-1}) of resistant *Enterococci* spp. from wild life species and cattle

Antibiotics/Animal	Vancomycin	Ampicillin	No antibiotic
Buffalo	$0{\cdot}21 \pm 0{\cdot}23^{a}$ (1/35)	$0{\cdot}66 \pm 0{\cdot}26^{a}$ (4/35)	$3{\cdot}76 \pm 0{\cdot}34^{a}$ (35/35)
Zebra	$0{\cdot}60 \pm 0{\cdot}19^{a}$ (8/40)	$0{\cdot}55 \pm 0{\cdot}21^{a}$ (4/40)	$3{\cdot}85 \pm 0{\cdot}28^{a}$ (40/40)
Wildebeest	$0{\cdot}24 \pm 0{\cdot}2^{a}$ (1/40)	$0{\cdot}22 \pm 0{\cdot}22^{a}$ (2/40)	$4{\cdot}35 \pm 0{\cdot}29^{a}$ (40/40)
Cattle	$0{\cdot}00^{a}$ (0/20)	$0{\cdot}00^{a}$ (0/20)	$2{\cdot}29 \pm 0{\cdot}51^{b}$ (20/20)

Groups with the same superscript were not statistically different. Numbers in brackets show how many animals out of tested that had CFU above the detection level of 100 CFU g^{-1}. Samples below this level were assigned a value of 1 CFU g^{-1} for statistical purposes.

Table 3 Species composition of *Enterococcus* isolates

Source	*Enterococcus faecium*	*Enterococcus faecalis*	*Enterococcus gallinarum*	*Enterococcus hirae*	Total
Buffalo (*N* = 29)	23	2	0	4	29
Wildebeest (*N* = 37)	29	3	2	3	37
Zebra (*N* = 40)	30	3	5	2	40
Cattle (*N* = 14)	13	1	0	0	14
Total (%)	95 (79·2)	9 (7·5)	7 (5·8)	9 (7·5)	120

Table 4 Antibiotic susceptibility of *Escherichia coli* from wild life in NCA and MNP

AB	Group	NCA	MNP	*P* Value*	BFL	WLB	ZBR	*P* value*
TE	R	16 (24·6)	23 (42·6)	0·030	20 (64·5)	8 (23·5)	11 (26·8)	0·0002
	I/S	49 (75·4)	31 (57·4)		11 (34·5)	27 (76·5)	30 (73·2)	
STX	R	17 (25·8)	15 (27·8)	0·957	13 (41·9)	11 (32·4)	8 (19·1)	0·0335
	I/S	49 (74·2)	39 (72·2)		18 (58·1)	23 (67·4)	34 (70·9	
CTX	R	13 (19·7)	9 (16·7)	0·904	9 (29)	8 (23·6)	5 (11·9)	0·231
	I/S	53 (80·3)	45 (83·3)		22 (71)	26 (76·4)	37 (88·1)	
CN	R	4 (6·1)	15 (27·8)	0·0003	1 (3·2)	7 (20·6)	10 (23·8)	0·050
	I/S	62 (93·9)	39 (72·2)		30 (96·8)	27 (79·4)	32 (76·2)	
AMP	R	32 (48·5)	25 (46·3)	0·352	12 (38·7)	20 (58·8)	20 (47·6)	0·375
	I/S	34 (51·5)	29 (53·7)		19 (61·3)	14 (41·2)	22 (52·4)	
ENR	R	3 (4·6)	23 (42·6)	<0·0001	8 (25·8)	10 (29·4)	8 (19)	0·295
	I/S	63 (95·4)	31 (57·4)		23 (74·2)	24 (70·6)	34 (81)	
AMC	R	10 (15·2)	9 (16·7)	0·549	9 (29)	5 (14·7)	5 (11·9)	0·236
	I/S	56 (84·8)	45 (83·3)		22 (71)	29 (85·3)	37 (88·1)	

STX, Sulphamethoxazole/trimethoprim; CTX, cefotaxime; TE, tetracycline; AMP, ampicillin; CN, gentamycin; ENR, enrofloxacin; AMC, amoxycillin-clavulanic acid; R, resistant; I, intermediate; S, sensitive; NCA, Ngorongoro Conservation Area, wild life only; MNP, Mikumi National Park; BLF, buffalo; WLB, wildebeest; ZBR, zebra.
**P*-value for the nil-hypothesis that the groups are not significantly different.

Table 5 Antibiotic susceptibility of *Enterococci* spp. from wild life in NCA and MNP

AB	Group	NCA	MNP	*P* value*	BFL	ZBR	WLB	*P* value*
TE	R	29 (42)	18 (30·5)	0·338	15 (44)	15 (36·6)	10 (26·3)	0·294
	I/S	40 (58)	41 (69·5)		19 (56)	66 (63·4)	28 (73·7)	
STX	R	22 (31·9)	14 (23·7)	0·015	10 (29·4)	14 (34·2)	8 (21·1)	0·625
	I/S	47 (68·1)	45 (76·3)		24 (70·6)	27 (65·8)	30 (78·9)	
RD	R	29 (42)	28 (47·5)	0·172	19 (55·9)	19 (46·4)	15 (39·5)	0·329
	I/S	40 (58)	31 (52·5)		15 (44·1)	22 (53·6)	23 (60·5)	
E	R	26 (37·7)	21 (35·6)	0·774	14 (41·2)	18 (43·9)	10 (26·3)	0·619
	I/S	43 (62·3	38 (64·4)		20 (588)	23 (56·1)	28 (73·7)	
CN	R	24 (34·8)	15 (25·4)	0·363	10 (29·4)	12 (29·3)	13 (34·2)	0·897
		45 (65·2)	44 (74·6)		24 (70·6)	29 (70·7)	25 (65·8)	
AMP	R	16 (23·2)	17 (28·8)	0·554	12 (35·3)	9 (22)	10 (26·3)	0·157
	I/S	53 (76·8)	42 (71·2)		22 (64·7)	32 (78)	28 (73·7)	

STX, sulphamethoxazole/trimethoprim; TE, tetracycline; AMP, ampicillin; CN, gentamycin; E, erythromycin; RD, rifampicin; R, resistant; I, intermediate; S, sensitive; NCA, Ngorongoro conservation area, wild life only; MNP, Mikumi National Park; BLF, buffalo; WLB, wildebeest; ZBR, zebra.
**P*-value for the nil-hypothesis that the groups are not significantly different.

compared to MNP, both expressed in absolute numbers and relatively to 16s rRNA copy number. The flora of cattle and buffalo was shown to contain a much higher proportion of *tetW* genes compared to wildebeest and zebra, and in particular wildebeest had a low number of this gene.

Table 6 $Log_{10} \pm$ SE gene copy numbers g^{-1} faecal sample from wild ungulates and zebu cattle

Sources and animal spp	*N**		*sulII* % of 16s rRNA		*tetW* % of 16s rRNA	16 rDNA
Mikumi	49	5·59 ± 0·10[a]	0·36 ± 0·30[a]	6·08 ± 0·12[b]	0·56 ± 1·2[b]	8·84 ± 0·10[b]
Ngorongoro	56	5·39 ± 0·10[a]	0·41 ± 0·27[a]	7·14 ± 0·11[a]	5·32 ± 1·14[a]	9·49 ± 0·10[a]
Zebra	40	5·61 ± 0·12[a]	0·12 ± 0·31[a]	6·24 ± 0·14[b]	2·74 ± 1·37[b]	8·92 ± 0·11[b]
Wildebeest	32	5·54 ± 0·13[a]	0·47 ± 0·37[a]	6·44 ± 0·13[b]	0·79 ± 1·49[b]	9·19 ± 0·12[ba]
Buffalo	33	5·30 ± 0·13[a]	0·63 ± 0·36[a]	7·29 ± 0·15[a]	4·31 ± 1·47[ba]	9·51 ± 0·13[a]
Cattle	20	5·94 ± 0·21[a]	0·43 ± 0·45[a]	7·14 ± 0·21[a]	7·90 ± 2·40[a]	9·742 ± 0·17[a]

*Number of samples analyzed.

Super script a,b,c: Numbers/proportions within each column bearing same letter are not significantly different at $P < 0·05$.

Discussion

Wild animals, particularly species that live in close association with humans, may be exposed to resistant bacteria in their environment, and antimicrobial resistance has been detected in faecal bacteria from a variety of wild animals, including birds, reptiles, mammals and fish, throughout the world (Rolland *et al.* 1985; Sayah *et al.* 2005; Jardine *et al.* 2012). In the current study, a quit high numbers of antimicrobial resistant *E. coli* and *Enterococcus* spp. strains were observed from some free-ranging buffalo, zebra and wildebeest. Included in this was *E. coli* showing resistance to cephalosporin, and showing synergy with clavulanic acid, which is the classical test for ESBL production. Further studies are indicated to elucidate which ESBL genes are carried by these isolates. Also the study demonstrated the presence in wild life of flouroquinolone resistant *E. coli*, and enterococci with resistance to VA or AMP.

Our observations cannot be explained by direct exposure to antibiotics, as the wild life authorities maintain a strict policy of none interference with wild life. Co-habitation with livestock in grazing and drinking water points was therefore hypothesized to be a contributing factor, and we designed this study to investigate whether such interaction was a risk factor for carriage of antibiotic resistant bacteria in wild life. We did not observe a general higher level of antibiotic resistant bacteria in wild life from NCA, where such interaction is taking place, compared to MNP where wild life does not interact with cattle. The results suggested that the practice of co-grazing did not results in frequent transmission of resistant bacteria or resistance genes from livestock to wild life ungulates, however, a larger study with more samples will be required to quantify the risk. One possible confounding factor is that in years with drought, animals in MNP may move out of the park boundaries where they may get in contact with livestock and humans. Bacteria obtained during grazing under these conditions may persist in the intestine and be a reason for the presence of resistance. Taken to its full consequences, the observation of low transmission of resistant bacteria may indicate that disease transmission between cattle and wild life may not be a major problem for disease-causing agents that require faecal-oral transmission, unless the infective dose is very low. However, the sensitivity of the sampling used in the current investigation was low, and further studies are indicated.

A possible explanation for the presence of antimicrobial resistant bacteria in Buffalo and Zebra is the behaviour of these animals. As has been reported for baboons (Rolland *et al.* 1985), they are frequently found in close proximity of staff houses, offices and lodges. It is possible that they come in contact with human refuse or even human excretes. Wildebeest on the other hand are much more timid animals and are afraid of human interactions. In NCA, Masai herders move with cattle and during this time, access to toilets is limited. Frequently bushes are used at time of urgency, and through this practice, wild animals may come into contact with human faeces. In MNP, however, such a situation does not exist. All present lodges for tourists in the two parks have septic tanks and soak pits. Flooding of septic tanks during raining season can occur and lead to contamination of grazing land, which may cause transfer of antibiotic resistant bacteria to the animals.

It is well known that colonization of the intestinal tract by resistant bacteria can occur even in the absence of selection pressures, particularly as a result of contact with resistant bacteria in food or on environmental fomites (Adesiyun and Downes 1999; Wright 2010). Antimicrobial resistant bacteria are also found in high numbers of soil samples (Riesenfeld *et al.* 2004), and from here they can be ingested during grazing and transfer their genes to the bacteria we have investigated. We cannot rule out that some of the resistance genes responsible for the phenotypes we have observed originate in soil bacteria. Finally, there is a magnitude of flying birds in both NCA (over 500 species) and MNP (more than 400 species) (TANAPA, 2013) and around natural and artificial

constructed water points, these come into contact with mammals. Healthy wild scavenging birds have been found often to carry high numbers of antibiotic resistant bacteria (Radhouani *et al.* 2009, 2012). These birds could also be responsible of spreading resistant bacteria from one point to another (Adesiyun and Downes 1999).

Grazing behaviour of wild animals differ from that of cattle. They cover a large area within the NCA compared to that of cattle. They also cross the Tanzanian border and interact with other wild animals and cattle in Kenyan country. In this scenario, it is possible for wild animals to feed on different vegetation (Ushimaru *et al.* 2007) and get contact with more environments that have been contaminated by birds and animal faeces containing resistant bacteria (Adesiyun and Downes 1999), and this may explain our observations. A more theoretical explanation is that plants rich in a variety of secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids may select for antibiotic resistance, since they have been found *in vitro* to have antimicrobial properties (Cowan 1999; Lewis and Ausubel 2006), However, further studies are needed to investigate whether they share resistance mechanisms with antibiotics.

Antibiotic resistant levels were found to be higher in wild animals compared to cattle, even though these are occasionally treated with antibiotics. A weakness of this study is the lack of data on consumption of antibiotics in these cattle. Such data do not exist and cannot be readily obtained due to lack of records. A recent study on antibiotic use in Tanzania showed that cattle are generally treated with oxytetracycline and sulphonamides as the most common drugs, and that use of cefalosporin and flouroquinolones cannot be afforded by the poor cattle keepers (Katakweba *et al.* 2012). Hence, the presence of indicator bacteria resistant to such drugs is more likely to be caused by interaction with humans than with cattle.

Analogous to our investigation, Rwego *et al.* (2008) conducted a study to see if habitat overlap can increase the risks of anthroponotic and zoonotic pathogen transmission between humans, livestock, and wild apes and found that contact with humans and/or livestock was indeed a risk factor for transmission of bacteria. *E. coli* were collected from humans, livestock and mountain gorillas (*Gorilla gorilla beringei*) in Bwindi impenetrable National Park, Uganda. Gorilla populations that overlapped at high rates with people and livestock in their use of habitat harboured *E. coli* that was genetically similar to *E. coli* from those people and livestock. The strains obtained from such animals were resistant to at least one antibiotic used by local people, and the proportion of individual gorillas harbouring resistant isolates declined across populations in proportion to the decreasing degrees of habitat overlapping with humans. We have not performed genetic fingerprinting of the strains obtained in this study, for the reason that too few colonies were obtained from each animal; with so few isolates it is very unlikely that we will detect genetically similar strains.

To investigate whether resistance levels in general differed between species and between the two groups of wild animals, i.e. those grazing with and those grazing without cattle, quantification by culture independent qPCR was performed for one common tetracycline resistance gene and one sulphonamide resistance gene. qPCR has previously been used in several investigations to quantify levels of resistance genes (Pei *et al.* 2006; Momtaz *et al.* 2012). The chosen gene *tetW* is chromosomally encoded and common in Gram positive bacteria (Scott *et al.* 1997). It has also been shown to be common in cattle (Peak *et al.* 2007), and it is present in the human gut flora (Scott *et al.* 2000). *sulII* represents the most common sulphonamide resistance genes in *E. coli* from many species of animals and in man (Trobos *et al.* 2008). No significant increase in gene pools were associated with co-grazing with cattle, supporting our observations from phenotypic based analysis, but in contrast to that investigation, the level of resistance (both expressed as absolute numbers of genes and number of genes relative to 16s rRNA) was not higher in wild life compared to cattle, suggesting that the phenotypic tests highlight differences due to other genes that the two investigated by qPCR.

In conclusion, the results of this study have demonstrated presence of antibiotic resistant *E. coli* and enterococci in wildlife animals in the absence of antibiotic pressure. Wild animals grazing together with cattle that are actively treated with antibiotics did not generally show higher levels of resistant bacteria than wild animals not grazing with cattle. In the context of this study, this indicates that co-grazing may not result in frequent transmission of resistant bacteria.

Acknowledgements

The government of Denmark through DANIDA is acknowledged for financial support to this study (grant number 66-08-LIFE). We owe much thanks to MNP and NCA staff who assisted in sample collection. We thank Henry Kissinga (Field and Laboratory work) and Mariam Kitojo (laboratory work) of Sokoine University of Agriculture Tanzania and Tony Bønnelycke, Pia Mortensen and Ljudmila Trojanova (laboratory work), University of Copenhagen for their invaluable help in the laboratory work.

Conflict of Interest

No conflict of interests declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Standard curve for quantification of the resistance gene *sulII*.

Figure S2. Standard curve used for quantification of resistance gene *tetW*.

Figure S3. Standard curve used for quantification of 16s rRNA gene copies.

Table S1. Resistant coliform from wild life species and cattle expressed relatively (%) to the total number of coliform bacteria in the same animal (numbers shown in brackets are number of animals out of total number of animals with resistant bacteria above the detection limit).