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SUMMARY

Wild-animals can act as reservoirs for resistant bacteria and transfer of resistance genes in the environment. These genes can spread to livestock and human either directly by transmission of shared resistant bacteria, or by horizontal gene-transfer to environmental bacteria. To ascertain at what extent wild-animals carry resistance genes, eight faecal samples from buffalo, zebra and wildebeest from Ngorongoro Conservation Area (NCA) and Mikumi National Park (MNP), and four control samples from local zebu cattle grazing together with wildlife in NCA. The qPCR was carried on 14 antimicrobial resistance genes including tetracycline (*tet(A), tet(B), 93 tet(C), tet(M), tet(O), tet(W),* macrolide, lincosamide, streptogramin B (*ermB, ermF*), sulphonamide (*sulI, sulII*), beta-lactam (*blaCTX-M-1 group, blaCMY-2, blaSHV*) and glycopeptide (*vanA*). Samples from NCA, both wildlife and cattle were positive for 8 out of 14 resistance genes. The most prevalent genes were *tet*(W) and *bla*$_{\text{CMY-2}}$ with the latter being of concern in encoding ESBL-type resistance. Three samples from Buffalo not interacting with cattle in MNP, were positive for *tet(W)* and blaCMY-2, and in addition for s*ulI.* This suggests that wild ungulates on savannah, irrespective of contact with cattle, may constitute a reservoir for antimicrobial resistance determinants. Further studies are indicated to determine resistance gene-pool among wildlife animals.

Keywords: Cattle, Wildlife, Antimicrobial resistance, qPCR, CMY-2

INTRODUCTION

Antibiotic resistance in human and animals has been described both in developed and developing countries (Katakweba *et al*., 2018). Bacteria with this characteristic can be isolated from a variety of environments, both in nature and agricultural settings (Koike *et al.* 2007; Costa *et al.* 2008; CheeSanford *et al.* 2009; Poeta *et al.* 2009; Poeta *et al.* 2010; Koike *et al.* 2010; Anyanwu *et al*., 2012; Goncalves *et al.* 2013; Arnold *et al*., 2016). Interaction between humans, livestock, wild-animals and their environment may cause exchange of antimicrobial resistant bacteria and their genes within and between these groups (Katakweba *et al*., 2015). Human activity in natural environments is increasing as population grow, ultimately narrowing the human-wildlife proximity (Skurnik *et al.* 2006; Pesapane *et al*., 2013, Katakweba *et al*., 2015). Unfortunately, the close proximity facilitates exchange of infectious diseases between humans and wildlife (Skurnik *et al*., 2006; Benavides *et al.* 2012; Pesapane *et al*., 2013; Carroll *et al*., 2015). In relation to this, Skurnik *et al*. (2006), found a correlation between the level of exposure to humans, his activities, and the prevalence of antibiotic resistance in bacteria from wildlife. Also, it has been implied that wild-animals can act as reservoirs for resistant bacteria consequently facilitating antibiotic resistance gene transfer throughout the environment (Dolejska *et al.* 2007; Katakweba *et al*., 2015; Arnold *et al*., 2016). Studies by Rolland *et al*. (1985), Rwego *et al*. (2008), Literak *et al*. (2009) and Pesapane *et al*. (2013), described antimicrobial resistance in African wild primates, mountain gorillas, wild boars and mongoose that were shared the same environment with humans.

In Ngorongoro Conservation Area (NCA), Tanzania, Maasai shepherds migrate with their short-horned zebu cattle, goats and sheep, donkeys and dogs interacting with wildlife species such as wildebeest (*Connochaetes taurinus)*, zebra (*Equus burchelli)* and buffalo (*Syncerus caffer)* through grazing and gathering at water holes together (Voeten and Prins 1999; Charnley 2005; Katakweba *et al*., 2015). Distant to the south from NCA is Mikumi National Park (MNP) where grazing is prohibited within park boundaries. It is therefore assumed that wildlife in MNP have limited contact with cattle, if any. A study by Katakweba *et al*. (2015), found *tet*(W) and *sulII* genes in the same animal species. We hypothesize that, if the samples were positive for two antibiotic resistance genes it is likely that there are more genes, and identical genes will be present in both wildlife and cattle samples. Therefore, the aim of this study was to find out if these animals harbor other resistant genes other than *tet*(W) and *sulII* that were mentioned by Katakweba *et al*. (2015), and to estimate whether these animals carry resistance genes in levels which can be considered a significant public health risk.

MATERIALS AND METHODS

Permission and study area

The study permission for this study was granted by Tanzania Commission for Science and Technology (COSTECH) (permit No. 2010-324-NA-2010-161).

The cross sectional study was carried out in NCA, Tanzania (GPS coordinates 3°12’ S 35°27’ E/3.209°S 35.46) and in MNP (GPS-coordinates are 7°12’S 37°08’E/ 7.200°S 37.13) (Fig. 1) (Katakweba *et al*., 2015). In the study area, wildlife (wildebeest, zebra and buffalo) and cattle residing in NCA were involved, while in MNP samples were only collected from wildlife (wildebeest, zebra and buffalo). Cattle were involved in NCA because they use the same grazing environment used by wildlife (Katakweba *et al*. 2015).

Faecal samples collection

The samples (Table 1) were collected from animals of interest in NCA (zebu cattle, buffalo, wildebeest, and zebra) and MNP (wildebeest, zebra and buffalo). The animals were located and followed while grazing. Once feces were voided, the sample was immediately collected from the middle section to ensure that it had not been in contact with the ground, put into a cooling box containing ice and transported to the Institute of Pest Management laboratory for DNA isolation within 24 hours (Katakweba *et al*., 2015)**.**

Table 1: Characteristics of sampling sites

Sampling site	Animal species	Laboratory number	± Cattle interaction
Ngorongoro CA	Zebu cattle	N22	+
Ngorongoro CA	Zebu cattle	N23	+
Ngorongoro CA	Zebu cattle	N43	+
Ngorongoro CA	Zebu cattle	N54	+
Ngorongoro CA	Buffalo	N21	+
Ngorongoro CA	Buffalo	N58	+
Ngorongoro CA	Zebra	N1	+
Ngorongoro CA	Zebra	N5	+
Mikumi NP	Buffalo	M3	-
Mikumi NP	Buffalo	M13	-
Mikumi NP	Buffalo	M15	-
Mikumi NP	Wildebeest	M16	-

CA = Conservation Area, NP= National Park, Interaction status (+ Interaction; - No interaction)

DNA extraction

Ten-fold dilutions from each sample were made in phosphate buffer saline (PBS). After thorough vortexing, 200 µl of the 10^{-1} dilution was used for DNA extraction using the QIA amp DNA Stool Mini Kit (Qiagen, Copenhagen, Denmark) according to the manufacturer's instructions. The DNA was eluted in 200 µl elution buffer and stored at -20°C and later on was transported to Denmark for resistance genes detection (Katakweba *et al* 2015).

Assays for antibiotic resistance genes and 16S rDNA

The DNA extracted from fecal samples was quantitatively analyzed for the presence of 14 antimicrobial resistance genes using qPCR as describe Schmidt *et al*. (2015). The following antibiotic-resistance genes were quantified by qPCR: tetracycline resistance genes *tet*(A), *tet*(B), *tet*(C), *tet*(M), *tet*(O), *tet*(W); macrolide, lincosamide, streptogramin B (MLS_B) resistance genes *ermB*, *ermF*; sulphonamide resistance genes *sulI*, *sulII*; beta-lactam resistance genes $bla_{CTX-M-1}$ group, bla_{CMY-2}, bla_{SHV} family and glycopeptide resistance gene *vanA*.

Furthermore, a qPCR assay targeting 16S rDNA was used to characterize the overall bacterial population size. The primers, conditions, and standard curves used for quantification are described elsewhere (Schmidt *et al.*, 2015). Each sample was tested in duplicate along with a positive control template in triplicate. Furthermore, a negative template control (NTC) (23 µl mastermix and 2 µl water) was also included.

RESULTS

With exception of *tet*(A) that was found in cattle only, wildlife and cattle samples in NCA were all positive for seven resistance genes namely *tet*(W), *ermF*, *sulI*, and *bla*$_{CMY-2}$., *tet*(M), *tet*(O), and *sulII*. Other fecal samples from wildlife and cattle were positive for one or more of the 14 antibiotic resistance genes tested. Samples from MNP, three from Buffalo were positive for *tet*(W) and *bla*$_{CMY-2}$, and one buffalo was further positive for *sulI*. Wildebeest was only positive to *tet(W)*. One Buffalo sample was the only sample that was negative for all the tested antibiotic resistance gene determinants. No samples from either sampling site were positive for *tetB, tetC, ermB, vanA, bla*$_{SHV}$ and *bla*$_{ctm-M-1}$.

The gene copy numbers g^{-1} feces for the different antibiotic resistance determinants and for 16S rDNA that was used as a proxy for the overall bacterial population size as shown in Table 2. Samples were found to contain resistance genes in the range of 10^3 to 10^6 copies per gram, feces were also detected in minimum 1 of the 4 cattle samples (Table 2). Variation of genes were observed within and between group of animals and their site of samples tested for the genes. *Tet(M)* was only detected in Cattle. For the *Tet (w),* genes were highest in Cattle followed bey Wildebeest, Zebra and Buffalo, *ermF* was highest in Zebra then Buffalo. In case of *SulI*, Buffalo was leading followed by Zebra and Cattle. Buffalo had highest *blaCMY-2* compared to Cattle and followed by Zebra. Finally, 16S rDNA was detected in all samples with highest range from Zebra, Buffalo and cattle. Based on the site's selection in this study, there was a difference between NCA and MNP whereby NCA having higher genes in all 14 genes studied.

DISCUSSION

The presence of antibiotic resistance genes did not depend on co-grazing cattle and wildlife, since wildlife samples from both locations did not differ markedly, despite restriction of cattle grazing in MNP and allowed in NCA. The presence of antibiotic resistance genes in areas where domestic animals are not grazing with wildtype suggest that wildlife are reservoir of resistance genes that are of public health importance and to the health of livestock population in Tanzania. These results were expected in NCA since samples from wildlife were collected in close proximity with grazing cattle. Also, it has been postulated that buffalo and zebra may exhibit similar grazing pattern as often found near staff housing, offices and lodges. Therefore, the buffalo and zebra may come in contact with human refuse or even human excretes (Rolland *et al.* 1985, Katakweba *et al.*, 2015: Mercat *et al.*, 2016), accounting for the antibiotic resistance gene detection in these samples.

A study by Mercat *et al*. (2016), in South Africa at Buffalo-Cattle interface, evidenced the dissemination of tetracycline, trimethoprim, and amoxicillin resistance genes (*tet*, *dfrA*, and *blaTEM-1*) in 26 isolated sub-dominant *E. coli* strains between nearby buffalo and cattle populations, that led them to hypothesize the role of the human-animal interface in the dissemination of genetic material from human to cattle and toward wildlife. Only one wildlife sample in the current study was negative for all the tested antibiotic resistance gene determinants. This sample also had a relatively low amount of 16S rDNA gene copies implying that a technical error might have happened. However, the internal quality control of the qPCR was within the accepted range.

The wide detection of the ESBL encoding *bla*$_{CMY-2}$ gene in quit high numbers in wildlife calls for concern. This gene is reported to be found on plasmids and encodes cephalosporinases that confer resistance to penicillins, beta-lactam-beta-lactamase inhibitor combinations, and cephalosporins (Li *et al.* 2007, Jorgensen *et al.* 2010). The plasmids are often associated with other multiple resistance genes and transposons, and it has been suggested that the *bla*$_{CMY-2}$ spread is both clonal and horizontal (Li *et al.* 2007, Agersø *et al.*2014). The location of the *bla*$_{CMY-2}$ in positive strains in the current investigation was not determined, but the number of samples that tested positive for the *bla*$_{CMY-2}$ gene (10 out of 12) was alarming.

A study in Denmark demonstrated that the *bla*$_{CMY-2}$ gene was present in samples obtained from broiler flocks where cephalosporin administration has been banned for 10 years (Agersø *et al.*, 2014), but no quantitative estimates were provided. This shows how the *bla*$_{CMY-2}$ gene can persist without the selection pressure induced by cephalosporin antibiotics. Our data suggests that wildlife might have a role as reservoir for antibiotic resistance genes, as previously suggested (Katakweba *et al*., 2015). We cannot rule out that the resistance genes that we have detected are carried in bacteria which just pass through the animals, however, the fact that they are present in so many wild animals and in relatively high numbers suggest that they are from bacteria, which are part of the normal flora. If these genes spread from nonpathogenic bacterial species in wildlife reservoir to a human or zoonotic pathogen, health consequences may erupt. Therefore, the finding of *bla*$_{CMY-2}$ among 10 of the 12 screened animals gives reason for further investigation of the antibiotic resistance gene prevalence among the wildlife, especially in areas with likelihood of interaction, directly or indirectly with livestock and humans. Furthermore, it should be noted that the present study only investigated a small number of samples collected from wildlife and cattle for the presence of 14 antibiotic resistance genes.

Wildlife animals at MNP are not interacting directly with cattle. Newer-the-less, resistance was observed in these samples, too. This resistance could arise through birds that migrate in and out of national parks, through contamination from humans living in or visiting the park, or ingestion of plants with antimicrobial properties as it has been suggested by Radhouani *et al*. (2013) and Katakweba *et al*. (2015). Based on these results, further studies should be conducted on antibiotic resistance gene-pool among wildlife in northern Tanzania in depth.

The copy of gene numbers per gram varied within and between wildlife groups and cattle. Also, there is a variation when compare with16S rDNA (Table 2), This could be explained by the fact that there was variation of DNA for qPCR gene detection. Cattle samples had higher gene numbers in *tet* and *sull* compared to wildlife and 16 rDNA. The same findings were reported by Katakweba *et al*. (2015) that the level of resistance (both expressed as absolute numbers of genes and number of genes relative to 16s rDNA) was not higher in wildlife compared to cattle, suggesting that the phenotypic tests highlight differences due to other genes.

CONFLICT OF INTEREST

No conflict of interest declared.

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Table 2 Resistance genes g^{-1} feces quantified in total DNA extracted from zebu cattle and wildlife from Ngorongoro Conservational Area and Mikumi National Park in Tanzania.

Genes/ Samples	**Gene copies g^{1} faeces**														
	tet (A)	*tet* (B)	*tet* (C)	*tet* (M)	*tet* (O)	*tet* (W)	*erm B*	*erm F*	*suII*	suIII	*bla*SHV Family	*bla* CTX-M-1	*bla* CMY-2	*van*A	*16S* rDNA
Cattle N22	0	0	0	2.57E+03	0	7.03E+04	0	1.27E+05	2.38E+03	0	0	0	2.25E+04	0	3.98E+09
Cattle N23	0	0	0	0	0	2.66E+05	0	1.27E+04	5.49E+02	0	0	0	3.23E+03	0	6.47E+09
Cattle N43	1.03E+04	0	0	0	6.29E+04	3.43E+05	0	1.09E+07	7.90E+04	2.42E+06	0	0	8.54E+03	0	2.01E+09
Cattle N54	0	0	0	0	1.05+04	1.58E+05	0	2.45E+04	2.46E+02	0	0	0	8.04E+03	0	1.91E+09
Buffalo N12	0	0	0	0	0	3.63E+03	0	2.31E+04	1.36E+02	0	0	0	6.44E+03	0	1.09E+09
Buffalo N58	0	0	0	0	0	1.76E+03	0	2.15E+04	4.23E+03	0	0	0	9.73E+03	0	2.02E=09
Zebra N1	0	0	0	0	0	5.55E+03	0	0	1.51E+03	0	0	0	6.27E+03	0	8.34E+08
Zebra N5	0	0	0	0	0	2.82E+03	0	8.17E+03	8.01E+02	0	0	0	7.69E+03	0	9.95E+08
Buffalo M3	0	0	0	0	0	4.72E+04	0	0	8.05E+03	0	0	0	3.83E+03	0	1.38E+09
Buffalo M13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.72E+07
Buffalo M15	0	0	0	0	0	3.87E+04	0	0	0	0	0	0	4.62E+03	0	5.01E+08
Wildebeest M16	0	0	0	0	0	5.93E+03	0	0	0	0	0	0	0	0	1.87E+08

Grey shading=antibiotic resistance genes levels were over the assays' limit of quantification. Unshaded numbers= resistance genes levels were over the assays' limit of detection.
0: not detected