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Molecular Identification and Antibiotic Susceptibility Patterns of Bacterial Isolates from Urine Samples of African Buffalo, Eland and Cattle

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Abstract

Presence of antibiotic resistant bacteria in natural environment is an escalating risk of serious implication on human and animal health. Livestock and wildlife have been long recognized as reservoirs for antibiotic resistant bacteria. Nonetheless, there is limited knowledge regarding the potential of livestock and wildlife urine to act as transmission corridor for the spread of antibiotic resistant bacteria. The present study aimed at evaluating antibiotic susceptibility patterns and molecular identification of bacteria isolated from livestock and wildlife urine samples. A total of 19 different bacteria isolated from urine samples of African buffalo, eland and cattle were subjected to antibiotic susceptibility test. The isolates showed diverse susceptibility patterns against co-trimoxazole, tetracycline, amoxycillin, ciprofloxacin, streptomycin, nalidixic acid, chloramphenicol and gentamicin. Of the tested isolates, 73.7 % were exhibited resistance while 31.6 % were intermediate to the range of antibiotics tested. High resistance prevalence to amoxicillin (58%), tetracycline (26 %) and co-trimoxazole (11%) by the tested bacteria was observed. This study reveals the bacteria associated with African buffalo, eland and cattle urine as potential candidates for antibiotic resistance. This information demonstrates the need for measures to be adopted to limit the presence of antibiotic resistant bacteria in wildlife and livestock reservoirs.

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Introduction

The burden of antibiotic resistance (ABR) continues to impact negatively on human health, animal production and peoples' livelihood (Ferri *et al.,* 2017). There is increasing rate of antibiotic resistance among humans and veterinary globally (Hawkey, 2008). This has raised fears for the potential start of second 'pre-antibiotic era' (Appelbaum, 2012). Antibiotic resistance has been rated by the World Health Organization as one of the top health burdens of the 21st century (WHO, 2007; Nolte, 2014). Even so, the situation has gotten even worse as resistance in most clinically important bacteria have become common (Giedraitienė, *et al.,* 2011). The current severity of ABR related infections, treatment failures and prolonged illness put pressure on global healthcare systems and financial burden to the

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affected families. The ability of ABR bacteria to transfer resistant genes, through horizontal gene transfer, causing resistance in susceptible bacteria and commensals has raised concerns (Von Wintersdorff *et al.,* 2016; Barlow, 2009). The resistant commensals have a potential to create a large resistance gene pool where resistant traits can be readily transferred to pathogenic bacteria (Landers *et al.,* 2012).

Recent statistics indicate that ABR wave is not only bound to hospital environment but also has emerged into livestock, wildlife and community acquired ABR (Ventola, 2015; Dias *et al.,* 2018). Livestock and wildlife have been profiled as one of the many breeding grounds for resistance to antibiotics (Vittecoq *et al.,* 2016). They provide a complex-multi host system where antibiotic resistant bacteria and genes get disseminated into the environments. The antibiotic resistant bacteria are acquired from contaminated environments, human sources as well as biology and ecology of the host (Arnold *et al.,* 2016; Vittecoq *et al.,* 2016). Also, the inappropriate use of antibiotics in agriculture to boost animal health and production has been cited as a factor for ABR in livestock and wildlife (Moyane *et al.,* 2013).

Animal and human health's are interdependent and bound to the health of the ecosystem in which they co-exist (Destoumieux-Garzón *et al.,* 2018). Such interconnection favours the spread and transmission of ABR bacteria and the flow of elements bearing antibiotic resistance genes. Some ABR bacteria and resistant residues are normally discarded in their biologically active form in urine and feces into natural resources (Oliver *et al.,* 2019). Therefore, disposal of animal waste in the environment is a potential platform for interactions that may lead to the development of variant forms of antibiotic resistance (Manaia, 2017; Juhas, 2015).

Regardless of its burden to animal and human health, the occurrence and susceptibility pattern of antibiotic resistant bacteria of wildlife and livestock origin is poorly understood (Dolejska and Literak 2019). As well, the knowledge on the role of wildlife and livestock in the dissemination of antibiotic resistant bacteria is scanty (Berendonk *et al.,* 2015). Understanding antibiotic resistance in livestock and wildlife is critical to human health because of the increasing importance of zoonotic diseases. As well, the knowledge can be used in predicting emergence of resistance among pathogens in specific environments. It is therefore important to understand antibiotic susceptibility patterns of bacterial isolates associated with wildlife and livestock urine.

Materials and Methods

Sampling Collection

Livestock (Bos taurus) and wildlife (Syncerus caffer and Taurotragus oryx) urine samples were collected at Kenyatta university Cattle farm (1.1767° S, 36.9365° E) and Kongoni Game Valley Ranch (0.7754° S, 36.3715° E) respectively. The later is located in a semi-arid agro-pastoralist ecosystem where livestock are allowed to co-graze with wildlife. The urine samples were collected opportunistically during translocation and des-naring activities as the animals urinated naturally. Universal bottles (50 ml) were used to collect the urine samples for two weeks. The collected urine samples were kept under refrigeration and transported to the Microbiology Laboratory-Kenyatta University for further analysis.

Isolation of Cultivable Bacteria from African Buffalo, Eland and Cattle Urine Portions

The collected urine portions for each animal were separately pooled into transparent plastic bottle and serial diluted. At dilution factor of 10-6, aliquots of 0.1 ml (100 µl) were pipetted aseptically and inoculated on the Cysteine-Lactose-Electrolyte Deficient (CLED) (Oxoid, Basingstokes, UK) in triplicates. The inoculated plates were then incubated for 24 hours at 37°C. On isolation, the bacteria colonies were further cultivated on Nutrient Agar (HiMedia, Mumbai India) for purification using streak plate method. The pure isolates were then grouped based on their morphological appearance as guided by Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). The isolates were thereafter preserved at −20°C on 20% glycerol stock (v v-1) for further analysis.

Molecular Characterization of Bacteria Isolated from African Buffalo, Eland and Cattle Urine

DNA Extraction and Polymerase Chain Reaction

Genomic DNA was extracted from 24 hour pure colonies using a DNeasy Blood & Tissue Kit (Qiagen, Hilden German) as guided by the manufacturer. The 16S rRNA gene region of the gDNA was amplified using 5'-AGAGTTTGATCMTGGCTCAG-3' (27-Forward) and 5'- CGGTTACCTTGTTACGACTT-3' (1492-Reverse) primers (Haas *et al.,* 2011). The Polymerase Chain Reaction was carried out in a 50 µL volume, containing 10 × PCR buffer (100 mM Tris [pH 8.3], 500 mM KCl, 4 mM MgCl2, 0.1% gelatin) (5 µL), 2 mM (2.5 µL) dNTP mixture, Taq DNA polymerase (1 µL), template DNA (1 µL), 2 µL each of primers and Dho (PCR water) up to 50 µL. The PCR reaction was done in Techgene thermocycler FTGENE5D model (Techne-UK). The reaction conditions were: pre-denaturation for 5 minutes at 94oC, 36 cycles of denaturation for 1 minute at 94oC, annealing for 1 minute at 54oC and extension for 2 minutes at 72oC and finally extension for 10 minutes at 72oC. The resultant amplicons were stored at -20oC for further use.

Gel Electrophoresis and 16S Rrna Gene Sequencing

The gDNA and the amplicons were checked for quality by gel electrophoresis. Briefly, a 1X TBE buffer was prepared by mixing 5X TBE buffer (10 mL) with distilled water measuring 90 ml. A 0.8% and 1.2 % agar rose gel for gDNA and amplicons were prepared in that order. A 1µL ethidium bromide was added to each solution and mixed further. The solutions were then transferred into the acrylic gel tray. A 14-well comb was inserted into the gel and left to set for 30 minutes. After solidifying, the combs were withdrawn and the gel tray was placed in the buffer tank and submerged under a 0.5X TBE at a depth of 5mm.

A 2 µL of Bromophenol blue (loading dye) was mixed with 3 µL and 7 µL for amplicons and gDNA respectively on a strip of parafilm. At separate events, the mixtures were loaded into the wells of the gel. A 1Kb DNA ladder, for gDNA and 100-bp DNA ladder for amplicons (Thermo Fischer Scientific, UK) were loaded as molecular weight markers alongside the amplicons. After the gel-electrophoresis run, the gel trays were removed from the buffer tanks. The products were then visualized under UV trans-illuminator light and the gels photographed using a digital photograph

Purification of the amplicons was done using Exonuclease-Shrimp Alkaline Phosphatase reagents as advised by the manufacturer. Thereafter, the amplicons were sequenced using a BigDye Terminator cycle sequencing kit (Applied Biosystems, USA) following the manufacturers' instructions. The 27-Foward and 1492-Reverse primers for 16S

rRNA gene were used for sequencing. The sequences were created by Sanger (Capillary) sequencing via the AB1 3730 DNA Sequencer (Applied Biosystems, USA).

Screening Bacterial Isolates for Antibiotic Susceptibility

Antibiotic susceptibility test was done using Kirby-Bauer disk diffusion method on Muller Hinton agar (Biolab, South Africa) as guided by Clinical and Laboratory Standards Institute (CLSI, 2016). Commercially prepared antibiotics; amoxicillin (10 µg), tetracycline (30 µg), co-trimoxazole (25 µg), streptomycin (300 µg), gentamicin (120 µg), chloramphenicol (30 µg), nalidixic acid (30 µg) and ciprofloxacin (5 µg) were used to test the isolates. The 24 hours colony cultures were used to prepare suspension corresponding to 0.5 McFarland turbidity standards. Within 15 minutes of inoculating the MHA (Oxoid, South Africa) plates, commercially prepared antibiotic disks were firmly placed on the inoculated MHA plates using a 6-disk dispenser (Oxoid, South Africa). The plates were then incubated for 24 hours at 35°C. After incubation period, the diameter of the zones was measured to the nearest millimeter using a digital calliper (0-150 mm). Zone diameters were classified as resistant, susceptible or intermediate.

Data Analyses

The raw sequences were edited and consensus generated using Finch-Tv (Mishra *et al.,* 2010) and DNA Baser software (Zhang *et al.,* 2012) respectively. The sequences obtained were compared with sequences in the NCBI GenBank databases using BLAST program (Altschul *et al.,* 1997) and the closely related isolates were retrieved from the database. Antibiotic susceptibility mean of the zones of inhibition were subjected to ANOVA with significant difference determination among means using Tukey's Honesty Significant Difference test at $P \leq 0.05$. The analysis was done using Statistical Analysis System version 9.1 (SAS Institute, Cary, NC).

Results

Isolation and Characterization of Bacteria Isolates

A total of 151 isolates were isolated from the urine samples of African buffalo, cattle and eland. Of these isolates, 41 % were from the cattle urine sample while 32 % and 27 % originated from the cattle and eland urine samples respectively. The 151 isolates were then put into 19 groups on the basis of their morphological resemblance (Table 1).

Table 1. Morphological grouping of bacteria isolated from urine samples of African buffalo, eland and cattle

Isolate characteristic	Urine isolate groups																		
	i	**ii**	**iii**	**iv**	**v**	**vi**	**vii**	**viii**	**ix**	**x**	**xi**	**xii**	**xiii**	**xiv**	**xv**	**vvi**	**xvii**	**xviii**	**xix**
Elevation	r	r	f	r	f	r	f	r	r	f	f	f	f	r	r	f	f	r	f
Opacity	op	op	op	op	op	tp	op	tp	op	tp	op	op	tp	op	tp	tp	op	op	op
margin	e	i	i	e	i	e	i	e	e	e	i	i	e	e	e	e	e	i	i
colony shape	c	fm	c	fm	fm	c	c	c	c	c	c	fm	c	c	fm	c	c	fm	c
colony size	m	m	l	sm	l	m	l	pp	m	m	sm	sm	pp	m	sm	pp	sm	l	m
texture	mo	mo	d	d	mu	mu	mu	mo	wt	wt	mu	wt	mo	d	mo	mo	mu	mu	mo
NA colour	o	w	cw	cm	w	w	cm	w	w	w	cm	cm	w	cm	w	cm	cw	cm	cm
CLED colour	y	y	b	b	b	b	b	y	b	b	b	b	b	b	y	y	b	y	y
texture	mo	mo	d	d	mu	mu	mu	mo	wt	wt	mu	wt	mo	d	mo	mo	mu	mu	mo
RI	b1	b2	b3	b4	b5	b6	E7	E8	E9	E10	E11	E12	C13	C14	C15	C16	C17	C18	C19

The presumptive morphological identification of the bacteria isolates was further confirmed using molecular identification (Table 2). Molecular characterization based on 16S rRNA gene region showed that the bacteria belong to different species and strains. At approximately 1500 bp, the amplicons showed definite and appropriately sized band in all lanes when visualized in 1.2 % agar-rose gel. Alignment and analysis of sequences showed that the isolates closely relate to lineages of known bacteria. Of the 19 sequenced isolates, 14 different species belonging were revealed.

Table 2 Genetic characterization of the bacteria isolates of African buffalo, eland and cattle urine

Laboratory designation	Isolate identification± (Accession number)	Accession No. of the NCBI match	16S rRNA gene similarity (%)
b1	*Planococcus massiliensis* (MK123489)	NR 144714.1	99
b2	*Providencia rettgeri* (MK123491)	CP017671.1	99
b3	*Bacillus cereus* ISSFR-3F (MK123497)	CP018931.1	99
b4	*Bacillus pumilus* (MK123487)	CP018574.1	99
b5	*Bacillus cereus* MLY1 (MK123488)	CP024655.1	99
b6	*Psychrobacter alimentarius* (MK123490)	NZCP014945.1	99
E7	*Bacillus cereus* ATCC 4342 (MK123495)	CP009628.1	99
E8	*Enterococcus faecalis* KUB3006 (MK123496)	AP018538.1	90
E9	*Bacillus megaterium* (MK123504)	CP026736.1	99
E10	*Streptococcus agalactiae* (MK123503)	NC004116.1	100
E11	*Bacillus cereus* M3 (MK123502)	CP016316.1	99
E12	*Bacillus safensis* (MK123505)	CP015611.1	99
C13	*Morganella morganii* sub.sp. *morganii* (MK123498)	NC020418.1	99
C14	*Micrococcus luteus* (MK123499)	NC012803.1	99
C15	*Ochrobactrum pituitosum* (MK123500)	CP018782.1	92
C16	*Enterococcus faecalis* OG1RF (MK123492)	CP002621.1	99
C17	*Bacillus cereus* CMCC P0021 (MK123493)	CP011151.1	99
C18	*Bacillus amyloliquefaciens* (MK123494)	HG328253.1	99
C19	*Alcaligenes faecalis* (MK123501)	CP021641.1	99

Antibiotic susceptibility profiles of the 19 bacteria isolated from African buffalo, eland and cattle urine samples

Out of the total isolates, 14 exhibited antibiotic resistance levels (Figure 1). Isolates b1 (Providencia rettgeri) and C15 (Ochrobactrum pituitosum) showed resistance against amoxicillin and streptomycin. Isolates E7 (Bacillus cereus ATCC 4342), E9 (Bacillus megaterium), E11 (Bacillus cereus M3), C13 (Morganella morganii sub.sp. morganii) and C17 (Bacillus cereus CMCC P0021) showed resistance against amoxicillin and co-trimoxazole. Isolates b5 (Bacillus cereus MLY1), b6 (Psychrobacter alimentarius), E10 (Streptococcus agalactiae) and C14 (Micrococcus luteus) were resistant to amoxicillin. Isolates b4 (Bacillus pumilus) and C19 (Alcaligenes faecalis) were resistant against chloramphenical while isolates C16 (Enterococcus faecalis OG1RF) was resistant against gentamycin. Isolates b1 (Planococcus massiliensis) and C15 (Ochrobactrum pituitosum) showed intermediate reaction against tetracycline. Isolates b2 (Providencia rettgeri) and b6 (Psychrobacter

alimentarius) showed intermediate reaction against co-trimoxazole while isolates C13 (Morganella morganii sub.sp. morganii) and C16 (Enterococcus faecalis OG1RF) revealed intermediate reaction against chloramphenical. A unique trend was revealed whereby C16 (Enterococcus faecalis OG1RF) and E8 (Enterococcus faecalis KUB3006) showed different susceptibility levels against gentamycin and chloramphenical even though they belong to the same species. Overall, ABR bacteria were isolated in both the wildlife and livestock urine samples.

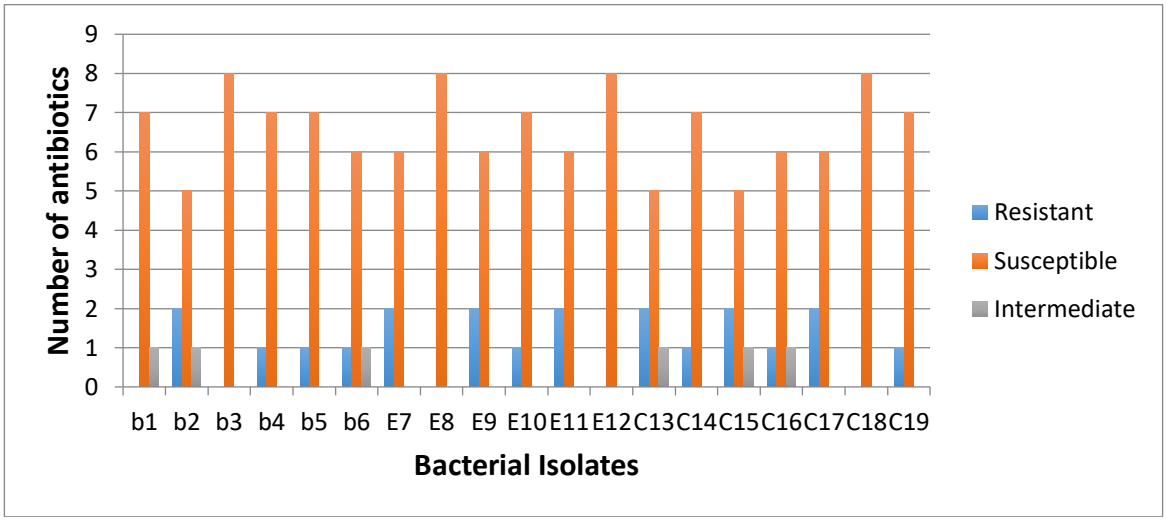

Figure 1. The averaged categorical antibiotic susceptibility profiles of the screened bacterial isolates against amoxicillin (10 µg), tetracycline (30 µg), streptomycin (300 µg), gentamicin (120 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), nalidixic acid (30 µg) and ciprofloxacin (5 µg)

The overall resistance towards the selected antibiotic tested was also determined. The isolates showed different susceptibility levels to the tested antibiotics. Amoxicillin showed the highest resistance prevalence at 58 % of the 19 tested isolates. This was followed by co-trimoxazole and streptomycine at 26 % and 11% respectively. Overall isolates resistance level towards chloramphenicol and gentamicin were 10.5 % and 5 % respectively (Figure 2).

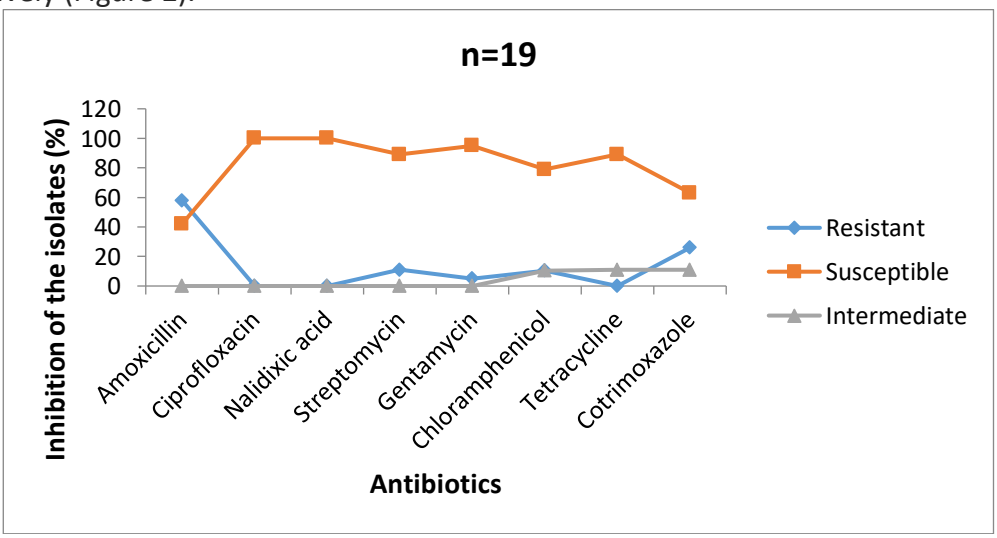

Figure 2: The averaged categorical performance of tested antibiotics against study bacteria. n = total number of tested bacterial isolates

Discussion

Morphological and molecular identification revealed presence of different species and strains of bacteria. The isolated bacteria most commonly exist as normal flora in animals. Also, some of these bacteria exist in nature including skin surface of the animals, soils air, plants and water. It is thus possible that the wildlife and livestock may have picked them horizontally during interaction with these environments. Members of Bacillus spp. were common in African buffalo, eland and cattle urine sampels. This may reflect the uniform distribution of these bacteria in the environment irrespective of the geographical location. It is likley that some of these bacteria may have evolved to adapt to different environments where they interact with wildlife and livestock and pose as agents of antibiotic resistance.

The antibiotic resistance expressed by Streptococcus agalactiae, Providencia rettgeri, Psychrobacter alimentarius, Morganella morganii sub.sp. morganii, Bacillus megaterium, Bacillus cereus CMCC P0021, Bacillus pumilus, Micrococcus luteus, Bacillus cereus MLY1, Alcaligenes faecalis, Ochrobactrum pituitosum, Bacillus cereus M3, Bacillus cereus ATCC 4342 and Enterococcus faecalis OGIRF depicts livestock and wildlife urine as a potential environmental reservoir of bacterial resistance. This result supports previous studies by Van den Honert *et al.* (2018), Aarestrup *et al.* (2015) and Vittecoq *et al.* (2016) who demonstrated that livestock and wildlife and the environments they impact can become significant reservoirs of ARB. Mercat *et al.* (2016) hypothesized that bacteria from wildlife that co-graze with livestock are likely to have ABR profile. This is in conformity with the current study where wildlife at Kongoni Game Valley Ranch co-grazes with livestock. Our study's findings further presents an evidence of resistant strains in livestock and wildlife urine which exposes the human population to the risks of antibiotic resistant elements.

The ability of different strains of Enterococcus faecalis to exhibit different susceptibility levels against gentamycin and chloramphenical could be related to source of the strain. Enterococcus faecalis OG1RF was isolated from cattle urine while Enterococcus faecalis KUB3006 was isolated from eland urine. Enterococcus faecalis OG1RF strain might have acquired from the environment genetic elements that code for antibiotic resistance against gentamycin and chloramphenical. The presence of resistant bacteria in the urine samples reflects not only possible spread of these bacteria between animals in the fields but also exchange of resistant components among the bacteria in the environment (Singer *et al.,* 2016).

The incidence of ABR bacteria in livestock and wildlife urine, as revealed in the present study, is alarming as the bacteria could disseminate ABR genes to other bacteria of human clinical significance (Woolhouse *et al.,* 2015). The origin of antibiotic resistance genes in clinical settings has been traced in natural environments (Mercat *et al.,* 2016). Wildlife and livestock that intermingles with humans are exposed to more antibiotic resistant phenotypes. They can therefore act as conduits for the dissemination of clinically relevant antibiotic resistance to the environment. Resistant bacteria isolates in urine could evolve into more harmful variants when exposed to a new environment. From there, they could be transferred back into the human and domestic animal environments, generating a host of major health issues in the future (Mercat *et al.,* 2016). Antibiotic resistance has been previously reported in bacterial isolates of livestock and wildlife origin (Jobbins and Alexander, 2015; Chee-Sanford *et al.,* 2009; Sørum and Sunde, 2001). However, to the best of our knowledge, this study is the first to report on the antibiotic susceptibility patterns of bacteria isolated from African buffalo, eland and cattle urine samples in Kenya.

Conclusions

These findings reveal the bacteria associated with African buffalo, eland and cattle urine as potential candidates for antibiotic resistance. The level of antibiotic resistance revealed in the present study forms a basis upon which intervention tools for monitoring the influence of wildlife and livestock on the development of ABR bacteria in the environment can be developed.

List of abbreviation

ABR: Antibiotic resistance; NCBI: National center for biotechnological information; BLAST: Basic local arrangement search tool; ANOVA: Analysis of variance

Declarations

Ethics approval and consent to participate

Permission to execute this research was approved by the National Commission for Science, Technology and Innovation (NACOSTI/P/17/73722/18052) Kenya. Informed verbal consent was obtained from animal handlers at Kongoni Game Valley Ranch and Kenyatta University

Consent for publication

Not applicable

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests

Funding

Not applicable

Authors' contributions

H.A.M., A.H., E.M.N. and J.N. developed the idea for the research and designed the experiment.

H.A.M. and L.M.L. collected the data and reviewed the manuscript. H.A.M. performed the experiments, analysed the data and prepared the manuscript. All authors read and approved the manuscript.

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