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OCCURRENCE OF SELECTED BACTERIAL ANTIBIOTIC RESISTANCE GENES IN SOILS OF ANIMAL FARMS IN UGANDA

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ABSTRACT The antibiotic resistance genes (ARG) of bacteria can be found in diverse environments such as the gut of mammals, soil, air, and water. In this research, we examined soils (for ARG) from 27 animal farms in Uganda (Mbarara, Wakiso and Mpigi districts) rearing poultry, pigs, dairy, or beef animals. Among these 27 places, there were antibiotic-free farms and those, which routinely used antibiotics to control diseases. DNA was extracted from soil samples using a commercial kit. A polymerase chain reaction (PCR) was performed to detect baScterial genes of resistance to sulphonamide (3 genes), beta lactam (1 gene), and Tetracycline (8 genes) antibiotics. The highest number of soils contaminated with these genes were from Mpigi district, whereas in Mbarara we found contamination of farm soils to a lesser extent. In all districts, the ARG were detected in farm soils regardless of the evidence of antibiotic usage; however, ARG were predominant in severe antibiotic consuming farms than the less consuming ones. Sulphonamide resistance genes were predominant in most district samples. In particular, the *sul2* (Sulphonamide) and *tetW* (Tetracycline) genes were the most prevalent in all samples, suggesting that fecal disposal or use of animal manure could drive the accumulation of ARG in these soils, making it a deadly reservoir, especially in areas with vast consumption of antibiotics.

Key Words: Bacteria; Antibiotic resistance; Soil resistome; Sulphonamide; Tetracycline.

INTRODUCTION

Survival of the human population majorly depends on agricultural products (FAO, 2017) and for purposes of sustaining production, a number of boosting applications are done in different farming systems, especially antibiotic usage for prophylaxis, growth promotion and controlling diseases in animals. Of major concern is the use of animal manure for improving soil fertility by most systems compared to synthetic fertilizers (Bayu et al., 2005). Animal wastes, such as chicken droppings, dung are dumped on farm soils, if no additional value is seen with them (not used on farm or sold as manure). However, the dumped or used refuse of antibiotic treated animals has been looked as a major anthropogenic driver of antimicrobial resistance at the animal human interface (Smith et al., 2019).

Continuous exposures of the gut microbiota to antimicrobials have yielded a positive

selection pressure on bacterial genomes so as to increase survival mechanisms giving rise to more resistant bacteria (Cheng et al., 2019). About more than half of antibiotics are not absorbed in the animal gut (Chee-Sanford et al., 2009). Theoretically, the continuous application of animal manure, can lead to many of the undigested antibiotics, along with bacterial antibiotic resistance genes (ARG) spreading into the environment via feces. In the long term, these human activities have affected the environment microbiomes, as a result of selective pressure induced by undigested antibiotic residues and gut ARG of animal origin (Wu et al., 2010). Thus, the increase of ARG in the environment could substantially contribute to the emergence of multidrug-resistant pathogens, which have been associated with antibiotic treatment failures in animals (Zhao et al., 2016).

Soil is one of the crucial reservoirs of microbial diversity, and the utilization of animal manure incriminates it to harbor a significant portion of the 'resistome', which is composed of Antibiotic Resistant Bacteria—ARB and their ARG (Udikovic-Kolic et al., 2014). The ARB are composed of the living bacteria in the soil, that are resistant to antibiotics, and are normally cultivated in media. The ARG consists of their genes, which are detected through molecular tools without cultivating them in or on media. The occurrence of these ARG is often regardless of history of antibiotic treatment on animals (Heuer & Smalla, 2007; Ferens & Hovde, 2011), however, the pathways that define the microbial community dynamics have not been fully explored. Different management approaches to agricultural production might have specific outcomes for soil microbial community composition, evolution, and development of antimicrobial resistance (Nõlvak et al., 2016). Studies on the distribution of ARG in farm soils have discovered independent patterns in different places (Li et al., 2017), including glacier environments (Segawa et al., 2013).

In Uganda, data on the approximate volume of antibiotics produced or used per year by the animal industry is scanty. Tetracyclines and sulphonamides are major antibiotic classes consumed globally and in the local Uganda context (Basulira et al., 2019; Okubo et al., 2019). The pharmacology of the two drugs shows that majority of the oral tetracyclines have a low absorption and shorter half-life than oral sulphonamides, thus, will be excreted to a more extent causing more selective pressure on the soil microbiota (Armalytė et al., 2019; Basulira et al., 2019). Also, studies have documented the use of soil fertilizers containing Sulphur compounds as a predictor for sulphonamides ARG in the environment (Nõlvak et al., 2016). Therefore, the spreading of animal manures can affect the antimicrobial resistance in the environment, especially on naïve bacteria that have not been exposed to antibiotics. The antibiotic usage in animal farms has played a role in the dissemination of antibiotic residues and evolution of microbial ARG in soils. Henceforth this study aimed to detect environment resistome outcomes caused by pollution of soil with animal refuse, focusing on the distribution of ARG from various livestock production systems in Uganda with varying antimicrobial usage practices.

MATERIALS AND METHODS

I. Soil Sampling

Soil samples were collected from animal farms that reared poultry, pigs, dairy, and beef animals in Uganda of which some of them were characterized by the utilization of antimicrobials (sulphonamide and tetracycline). Additionally, other antimicrobials such as ivermectin were used to control animal diseases. The details about the antibiotic and antimicrobial using are written in the Table 1. Basically, the authors had access only to the

Table 1. Soil sampling sites in Uganda and their respective characterizations

Sample ID	District	Geographic coordinate	Animal manure	Drug usage	General description
W1	Wakiso	0°23' 41" N 32°28' 20" E	Chicken feces	Tetracycline, Sulphonamide	Conventional chicken farm, soils containing chicken feces treated with antibiotic
W2	Wakiso	0°23' 41" N 32°28' 20" E	Chicken feces	Tetracycline, Sulphonamide	Conventional chicken farm, soils nearby chicken house (treated with antibiotic)
W3	Wakiso	0°23' 41" N 32°28' 20" E	Chicken feces	Tetracycline, Sulphonamide	Conventional chicken farm, soils with chicken feces treated with antibiotic
W4n	Wakiso	0°23' 41" N 32°28' 20" E	Chicken feces	Tetracycline, Sulphonamide	Conventional chicken farm, soils with spread chicken feces treated with antibiotic
W5	Wakiso	0°23' 21" N 32°28' 27" E	Pig feces	No Antibiotic[$]	Pig farm, soils nearby Pig's feces, non-treated with antibiotic
W7	Wakiso	0°23' 21" N 32°28' 27" E	Pig feces	No Antibiotic	Pig farm, soils nearby Pig's feces, non-treated with antibiotic
W8	Wakiso	0°23' 21" N 32°28' 27" E	Pig feces	No Antibiotic	Pig farm, soils nearby Pig's feces, non-treated with antibiotic
W9	Wakiso	0°23' 26" N 32°28' 26" E	Cattle feces	No Antibiotic	Soil from nearby Cattle's feces, non-treated with antibiotic
W10n	Wakiso	0°23' 26" N 32°28' 26" E	Cattle feces	Tetracycline, Sulphonamide	Conventional cattle farm, soils spread with Cattle's feces treated with antibiotic
W11	Wakiso	0°23' 26" N 32°28' 26" E	Cattle feces	Tetracycline, Sulphonamide	Conventional cattle farm, soils with Cattle's feces treated with antibiotic
M1	Mpigi	0°12' 17" N 32°18' 39" E	Pig feces	Tetracycline, Sulphonamide	Soils containing Pig's feces treated with antibiotic
M2	Mpigi	0°12' 17" N 32°18' 39" E	Chicken feces	Tetracycline, Sulphonamide	Soils nearby chicken house treated with antibiotic
M3	Mpigi	0°12' 17" N 32°18' 39" E	Cattle feces	Tetracycline, Sulphonamide	Soils nearby Cattle's feces treated with antibiotic
M4	Mpigi	0°09' 07" N 32°17' 25" E	Cattle feces	Tetracycline, Sulphonamide	Soils nearby Cattle's feces treated with antibiotic
M5	Mpigi	0°09' 07" N 32°17' 25" E	Chicken feces	Tetracycline, Sulphonamide	Soils containing Chicken's feces treated with antibiotic
M6	Mpigi	0°09' 07" N 32°17' 25" E	Pig feces	Tetracycline, Sulphonamide	Soils containing Pig's feces treated with antibiotic
M7	Mpigi	0°10' 44" N 32°18' 53" E	Pig feces	No Antibiotic	Soils containing Pig's feces non-treated with antibiotic
M8	Mpigi	0°10' 44" N 32°18' 53" E	Cattle feces	No Antibiotic	Soils rhizosphere containing Cattle's feces non-treated with antibiotic
M9	Mpigi	0°10' 44" N 32°18' 53" E	Chicken feces	No Antibiotic	Soils from nearby Chicken house non-treated with antibiotic
M10	Mpigi	0°10' 44" N 32°18' 53" E	Cattle feces	No Antibiotic	Soils containing Cattle's feces non-treated with antibiotic
MB1	Mbarara	0°36' 45" S 30°40' 01" E	Chicken feces	Drug free, red/hot pepper	Drug free, home made fermented foods, a red/hot pepper concoction to boost immunity
MB2	Mbarara	0°36' 45" S 30°40' 01" E	Pig feces	Drug free, red/hot pepper	Drug free, home made fermented foods, a red/hot pepper concoction to boost immunity
MB3	Mbarara	0°34' 25" S 30°32' 53" E	Pig feces	Ivermectin	Use ivermectin
MB4	Mbarara	0°34' 25" S 30°32' 53" E	Cattle feces	Ivermectin	Use ivermectin
MB5	Mbarara	0°34' 06" S 30°31' 21" E	Cattle feces	Red/hot pepper	a red/hot pepper concoction to boost immunity in case of chicken disease*
MB6	Mbarara	0°34' 06" S 30°31' 21" E	Chicken feces	Red/hot pepper	a red/hot pepper concoction to boost immunity in case of chicken disease
MB7	Mbarara	0°33' 42" S 30°32' 15" E	Pig feces	No treatment[&]	No veterinary interventions done

* Application of red/hot pepper as an alternative product to control diseases.
[$] Lack of antibiotic using to control diseases.
[&] Lack of disease control on animals.

name but not the amount of these products. These farms were located in central (Mpigi and Wakiso districts) and western Uganda (Mbarara district). The detailed description of each sampling site is written as well as their respective locations (Table 1). From each place, four 15 cm deep cores (sub-samples) were taken from the soil; 20 cm away from the animal manures, cages, and pens which were also randomly chosen in the areas and the cores combined into one sample. They were transported at room temperature and stored in a freezer (−18°C) for subsequent DNA analyses on the same day. The number of samples that were collected were 27: Wakiso (10), Mpigi (10), and Mbarara (7). Sampling was performed in September of 2017, a season characterized by high humidity and temperature ranging from 20–28°C.

II. DNA Extraction and Polymerase Chain Reaction Analyses

Genomic DNA was extracted from 0.25 g fresh weight of the well combined soil per sample using the Power Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The DNA concentration was measured using Thermo Scientific Nanodrop 2000 (Thermofisher Co., USA). Polymerase Chain Reaction (PCR) amplification was carried out in a 20 µl reaction mixture containing the 1 µl of genomic DNA from soil (around 20 ng/µl concentration), 10 µl of Emerald Amp MAX PCR Master Mix (Takara Co., Japan), 2 µl of each forward and reverse primers targeting the gene (0.4 µM), and 5 µl of sterilized distilled water. Twelve primers were used in this assay: sulphonamide (*sul1*, *sul2*, *sul3*) (Hoa et al., 2008), tetracycline (*tetM*, *tetO*, *tetS*, *tetW*, *tetC*, *tetA*, *tetB*, *tetL)*, and beta-lactamase (*blaTEM*) (Colom et al., 2003; De Vries et al., 2011; Kim et al., 2007). Amplification consisted of an initial denaturation for 3 minutes at 94°C followed by a cycle of 30 s of denaturation at 94°C, 1 minute annealing at 55°C (except for the *tetM*, *tetW*, *tetL* at 52°C), 1 minute elongation at 72°C repeated 35 times, and a final elongation step of 7 minutes at 72°C. The PCR products were subjected to electrophoresis in agarose gel (0.8%) and 1X TAE, and viewed under light after staining with Midori Green solution (Nippon Genetics Co, Japan) (1 µl per sample). The presence and absence of each gene was verified and recorded by visualization on the gel pictures and the bands at appropriated size as previously described (Segawa et al., 2013). Additionally, for the genes *tetA*, *tetB*, *tetC*, *sul1*, and *sul2*, the positive controls were also amplified and used as references; the other genes were verified by the band size as described by the literature. The negative controls were performed with only nuclease free water.

II. Data Analysis

The amplification results of 12 gene primers used on 27 soil samples to construct the heat map composed of 324 experimental units (EU). The presence and absence of bands generated ARG in PCR gels was recorded using 1 and 0 as representative values respectively to perform the Kruskal-Wallis test and the Principal Coordinate Analysis (PCoA) with the GENES software (Cruz, 2013). The PCoA was used to verify the effects of groups related to antibiotic usage and location.

RESULTS

In the current research, three districts in Uganda were surveyed to examine the presence of ARG in soils, where those genes related to sulphonamide resistance were predominant in

most of the samples in all districts. Nine samples were from soils that have been mixed with chicken, nine with pig, and nine with cattle dungs. Forty-four percent of the farms used antibiotics particularly tetracycline and sulphonamide derivatives and 33.3% had no history of antimicrobial consumption in the past six months. The heat map shows that ARG were detected 224 times (69%) among 324 PCRs. The detection of these genes was higher in Mpigi and Wakiso districts (37%), followed by Mbarara (26%). This last could be considered relatively free of sulphonamide in farms without antibiotics usage. In Wakiso, the ARG were detected in places with routine antibiotic usage. Although these genes could be found in most of the areas, the places without antibiotic usage history had less detection rates of ARG (Fig. 1).

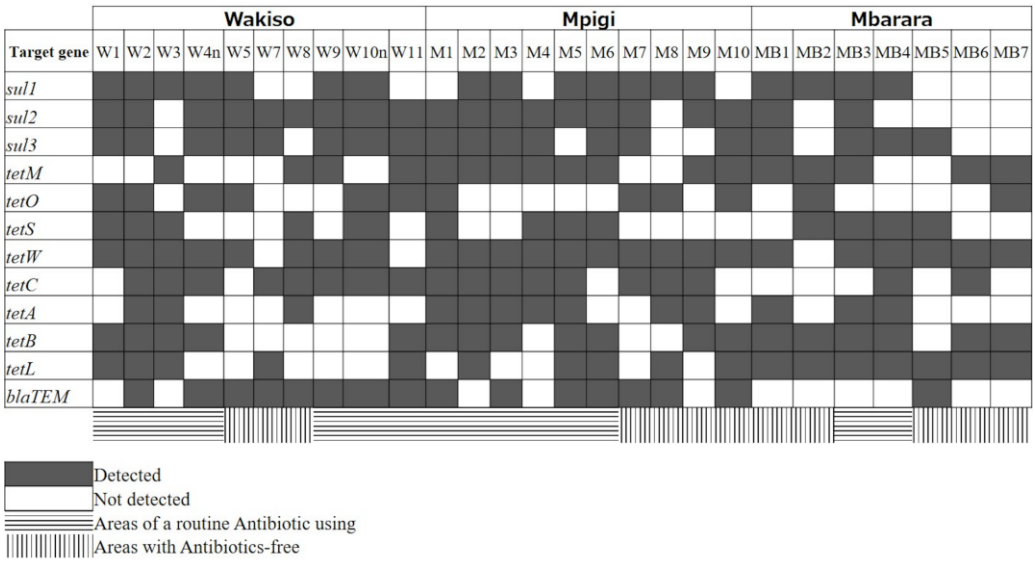

Fig. 1. Animals farm soils which the antibiotic resistance genes were detected

In poultry farms, the ARG were detected more within those that used antibiotics, followed by pig and cattle ones. In three places, found in Wakiso, Mpigi, and Mbarara, the absence of antibiotic use on pig farms lead to the lowest number of samples containing ARG. Additionally, the *blaTEM* gene was often detected in Wakiso (80.0%) and Mpigi (70.0%), however in Mbarara areas it was found in only one place, unexpectedly in an antibiotic-free area. In poultry farms, the ARG were strongly detected within those that used antibiotics. A similar pattern was found in pig and cattle farms. In three places, found in Wakiso, Mpigi, and Mbarara, the absence of antibiotic use on pig farms lead to the lowest number of samples containing ARG (Fig. 1). The presence of ARG could for some samples be distinguished by location and history of antibiotic usage. Especially Mbarara could clearly be separated from other locations. Similarly, in terms of antibiotic using, most of the samples could be separated (Fig. 2). However, the Kruskal-Wallis test indicated that these factors did not have significant effects on prevalence of most genes. More specifically, the significant effects were influenced by antibiotic usage (*tetS*), animals reared (*tetB*), and location (*sul2*, *blaTEM*) (Table 2).

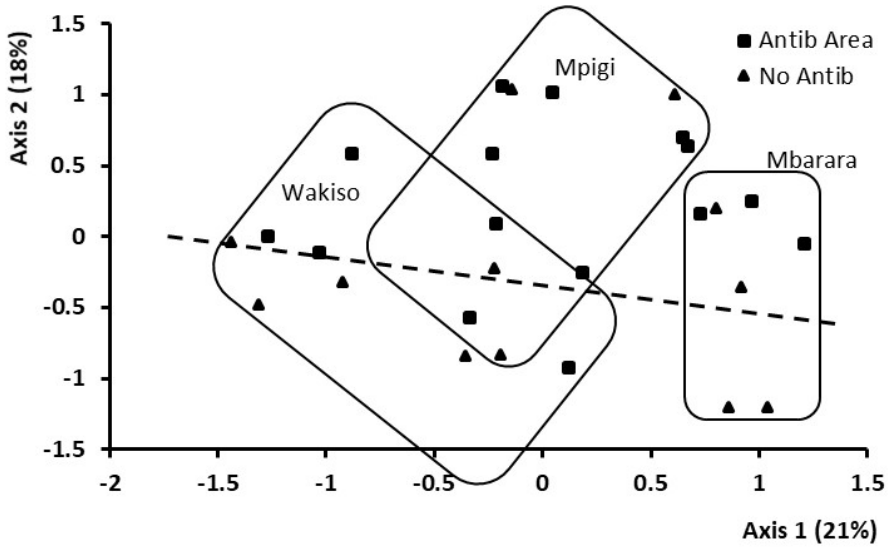

Fig. 2. Principal coordinate analysis of the presence of antibiotic resistant bacterial genes in soils of animal farms in Uganda

Table 2. Kruskal-Wallis test of the samples according to Antibiotic using (Yes or No), Animals reared (Poultry, Cattle, Pig) and Location (Wakiso, Mbarara, Mpigi)

	sul1	*sul2*	*sul3*	*tetM*	*tetO*	*tetS*	*tetW*	*tetC*	*tetA*	*tetB*	*tetL*	*blaTEM*
Antibiotic using	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Animals reared	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.
Location	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*

* Significant at 95%
** Significant at 99%
n.s. Non-significant

DISCUSSION

This is the first study in Uganda addressing the environmental impact of using organic manure from antibiotic treated animals in soils. The presence of ARG could partially suggest the history of antibiotics to control animal diseases, as well as their proliferation to other areas, including places without antibiotic usage. However, the ARG have been found in manure originating from antibiotic free animals (Udikovic-Kolic et al., 2014), suggesting the natural occurrence of bacteria harbouring ARG (intrinsic resistance) in the animals guts (Heuer & Smalla, 2007; Stanton et al., 2011).

Using conventional PCR methods, the study detected a few of the genetic determinants (ARG) of tetracyclines, beta lactams and sulphonamides in samples regardless of the farming system. The three antibiotic classes are vastly used in both human and veterinary medicine globally and locally as shown by our and previous findings (Basulira et al., 2019; Okubo et

al., 2019). The occurrence of more ARG in soils of farms with more antibiotic usage, as stated in the Fig. 1, unmasked an already existing relationship between antibiotic consumption and generation of ARG in the environment. More genes were detected in soils from farms of central Uganda districts (Wakiso and Mpigi). This could be attributed to a vast spectrum of activities linked with antimicrobial usage and misuse such as disease treatment, growth promotion, and prophylaxis as earlier demonstrated by Bashahun and Odoch (2015). Coupled to the latter, the heat map pronounces more antimicrobial consumption on the central Ugandan farms than the western ones. These areas could be considered as representative to evaluate the differences between those with antibiotic usage and those with no usage.

In this survey, farms with no usage of common antimicrobials contained some ARG such as *tetW* and *blaTEM*. This could have been due to importation or buying of manure from other contaminated farms, a practice usually done to sustain farming. The latter behavior threatens on-farm biosecurity endeavors and can influence ARG pathways in soils of farm settings (Armalytė et al., 2019).

The results were able to give mainly suggestive hypotheses linked with the environmental occurrence of ARG, because the sample size used was not powerful enough for the study to draw conclusions from a point of statistical inference. The soil microbiome is large (Armalytė et al., 2019) and still was not fully explored.

This preliminary study about the detection of ARG in soils gives baseline findings that suggest that human habits such as the usage of particular antibiotics and uncontrolled waste disposal on animal farms can affect their prevalence. In this case, the origin of manure in the fields was majorly from the animals at the farm; therefore the diversity of ARG in the soil could possibly be related to the antibiotic usage practices. This conclusion could be supported by the following observations: the PCR methods portrayed that ARG can be detected in the soils of farms with more antibiotic usage; commonly used antibiotics (tetracyclines and sulphonamides) had their ARG spotted in the environment. Further studies are also necessary for quantification and identification (Real Time PCR, Amplicon Sequencing) of these ARG, including advanced culture throw more insights into understanding the soil microbiome. These studies could lead to set up of some programs to control this environmental health problem.

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