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Phenotypic and genotypic analyses of antimicrobial-resistant bacteria in livestock in Uganda.

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Running title: Drug-resistant bacteria in Ugandan livestock

Abstract

Antimicrobial resistant bacteria (ARB) in livestock are a global public health concern, not only because they prolong infectious diseases but also they can be transferred from animals to humans via the food chain. Here, we studied ARB in livestock at commercial and subsistence farms (n = 13) in Wakiso and Mpigi districts, Uganda. We inquired from the farmers about the type and the purpose of antimicrobial agents they have used to treat their livestock. After collecting feces, we isolated antimicrobial-resistant *Escherichia coli* from livestock feces (n = 134) as an indicator bacterium. These strains showed resistance to ampicillin (44.8%), tetracycline (97.0%), and sulfamethoxazole-trimethoprim (56.7%). The frequency of ampicillin-resistance was significantly correlated with the usage of penicillins to livestock in the farms (P = 0.04). The metagenomics data detected 911 antimicrobial resistant genes that were classified into 16 categories. Genes for multidrug efflux pumps were the most prevalent category in all except in one sample. Interestingly, the genes encoding third-generation cephalosporins ($bla_{\text{CTX-M}}$), carbapenems (bla_{ACT}), and colistin (*arnA*) were detected by metagenomics analysis although these phenotypes were not detected in our *E. coli* strains. Our results suggest that the emergence and transmission of cephalosporin, carbapenem, and/or colistin-resistant bacteria among livestock can occur in future if these antimicrobial agents are used.

Keywords: antimicrobial resistant bacteria, *Escherichia coli*, livestock, metagenomic analysis, Uganda

Introduction

Emergence and distribution of antimicrobial-resistant bacteria (ARB) is a global public health concern (World Health Organization, 2014). ARB in food-producing animals are an important issue, not only for animal health and animal welfare but also public health, because ARB can be transmitted to humans via the food-chain (Marshall and Levy, 2011; WHO, 2014). ARB are also an economic burden for farmers because of costs incurred in treatment failure and prolonged period of treatment of the bacterial infections (Bengtsson and Greko, 2014). Antimicrobial agents have been used in livestock as a growth promoter, which may increase selective pressure on ARB (Marshall and Levy, 2011). National surveillance and monitoring system for ARB in livestock has been instituted in several countries (WHO, 2014). However, the frequency and occurrence of ARB in many Sub Saharan African countries are still unclear.

Escherichia coli is often utilized as an indicator bacterium for antimicrobial resistance of Gram-negative bacteria because it is a commensal bacterium in gut microbiota (Varga *et al.*, 2008). In addition, antimicrobial resistance in *E. coli* is important because it can cause diseases in both humans and animals. In veterinary medicine, *E. coli* causes infections such as mastitis in cows, diarrhea in calves, and colibacillosis in piglets and chickens. (Bradley, 2002; Dziva and Stevens 2008; Luppi 2017). Since antimicrobial-resistant genes are often encoded on transferable genetic elements such as plasmids, *E. coli* can easily receive antimicrobial-resistance genes from other bacteria via horizontal transfer, hence this can serve as a reservoir of resistance genes within gut microbiota (Bailey *et al.*, 2010; Carattoli 2008). Especially, extended-spectrum beta-lactamase (ESBL) and/or carbapenemase-producing *E. coli* is a major concern because it shows resistance against most of beta-lactam antimicrobial agents (Carattoli, 2008).

The Food and Agriculture Organization of the United Nations (FAO) estimates that the consumption of meat and milk in Africa will triple by 2050 (FAO, 2013); livestock production is an important agricultural sector in Africa. According to a surveillance data in 2005, livestock production in Uganda contributed 493 million US dollars per year which accounted for about 5.56% of total Ugandan GDP. (FAO, 2005). The 2014 FAO census data estimates that in Uganda there are 4.9 million cattle, 2.0 million pigs, 3.9 million sheep and goats, and 60.0 million poultry. (FAO2017). With the shortage of veterinarians in food animals in Uganda becomes a big problem of livestock disease control. For instance, in 2002, the ratio of veterinarian to livestock was 1: 10,500 livestock units (LSU; based on the number of cattle, goats, sheep, pigs and chickens) while it was 1: 2,255 LSU in the USA (African Development Fund, 2002; FAO 2017, OIE 2016). Interestingly, farmers in Uganda can purchase antimicrobial agents without the prescription of a veterinarian and even go ahead to treat their livestock. This therefore, predisposes to the emergence ARB due to improper use of antimicrobial agents in livestock (Queenan *et al.*, 2016).

In Uganda, several studies have detected and characterized ARB at a phenotypic level, for instance Afema *et al.* and Byarugaba *et al* detected antimicrobial-resistant *E. coli*, *Salmonella*, and *Enterococcus* spp. from livestock at a slaughterhouse (Afema *et al.*, 2016; Byarugaba *et al*, 2011), Kateete *et al.* detected antimicrobial resistant coliforms from milk samples with mastitis (Kateete *et al.*, 2013). However, these studies did not identify antimicrobial-resistant genes of their isolates. Here, we collected commensal *E. coli* from livestock feces derived from Ugandan farms to characterize antimicrobial susceptibilities and genotype of the isolates. In addition, we identified antimicrobial resistance genes contained in the feces by metagenomics analysis.

Materials and Methods

Sampling and isolation of *E. coli*

Fresh feces of livestock (cattle, pig, goat, and layer chicken) were collected at commercial farms (defined as farms that produce livestock products and/or live animals for market) and subsistence farms (that produce enough to feed themselves and their families) in Wakiso (n = 7) and Mpigi (n = 6) district in September 2016 and February 2017. Both districts are in the central region of Uganda, about 15 km (Wakiso) and 40 km (Mpigi) from Kampala, the capital of Uganda. When visiting the farms, we inquired from the farmers about (1) the type of livestock, (2) the number of livestock on the farm, and (3) the usage of antimicrobial agents. The bottles or pouch packs of antimicrobial agents were recorded when the farmers kept them. We also inquired health conditions of the livestock from the farmers and the district veterinary officers, and we collected feces from apparently healthy livestock only. A total of 130 fecal samples (n = 49 from Wakiso and n = 81 from Mpigi) were collected (Table 1). These samples were transported immediately to the Central Diagnostic Laboratory of the College of Veterinary Medicine, Animal resources and Biosecurity, Makerere University in Kampala, Uganda for further analysis.

Feces were diluted in 0.85% sterilized saline, and 1 mL of fecal solution was inoculated on a Petrifilm SEC plate (3M Company, MN, USA). After overnight cultivation at 37°C, up to four blue colonies which were suspected to be *E. coli* by β-gluclonidase activity were sub cultured on ES Colimark agar media (Eiken Chemical Co., Ltd., Tokyo, Japan) overnight at 37°C. Single colonies with blue color were defined as *E. coli* isolates. In addition, these isolates were checked by *E. coli*-specific PCR (Wang *et al.*, 1996).

Antimicrobial susceptibility testing

To characterize antimicrobial susceptibility of *E. coli* isolates, minimum inhibitory concentration (MIC) for 12 antimicrobial agents (ampicillin [AMP], cefazolin [CFZ], cefotaxime [CTX], gentamicin [GEN], kanamycin [KAN], tetracycline [TET], minocycline [MIN], nalidixic acid [NAL], ciprofloxacin [CIP], colistin [CST], chloramphenicol [CHL], and sulfamethoxazole-trimethoprim [SXT]) was tested by a broth micro-dilution method using frozen plates (Eiken Chemical) according to the manufacturer's instructions. *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains for the MICs. Isolates with higher MIC for antimicrobial agents than the MIC breakpoint on the Clinical and Laboratory Standards Institute (CLSI) guideline were defined as resistant, while breakpoint to CST was determined as ≥4 mg/L, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline (CLSI, 2016; EUCAST 2017). Isolates that showed resistance to at least one antimicrobial agent were used in further analyses. The correlation between the frequency of antimicrobial resistance and the use of respective antimicrobials on farms was analyzed by chi-squared test or Fisher's exact test using Microsoft Excel 2016 with Statcel v3 (OMS Ltd., Saitama, Japan) as an add-in for statistical analysis.

Phylogenetic typing

PCR-based phylogenetic typing for *E. coli* was performed (Clermont *et al.*, 2000). According to the PCR results, isolates were classified into seven groups and subgroups: A_0, A_1, B_1, $B2_2$, $B2_3$, D_1, and D_2 (Escobar-Páramo *et al.*, 2004).

Antimicrobial resistance genes in *E. coli* strains

PCR were performed to detect antimicrobial resistance genes as follows: for AMP-resistant strains, bla_{TEM}, bla_{SHV}, and bla_{OXA}; TET-resistant strains, *tetA, tetB, tetC, tetD, tetE,* and *tetG*; for SXT-resistant strains, *sul1* and *sul2* (Colom *et al.*, 2003; Phuong *et al.*, 2008; Vignaroli *et al.*, 2012).

Metagenomic analysis on shotgun DNA library

Metagenomic analysis were performed to detect antimicrobial resistance genes contained in feces. DNA was isolated from six randomly chosen representative fecal samples from Wakiso (cattle 1, pig 3, and layer 2). The Feces were collected in a storage buffer (Hayaishi and Kawamoto, 2006) at room temperature. DNA was extracted from fecal bacteria as described previously (Ushida *et al.*, 2016). Briefly, fecal pellets were washed with phosphate-buffered saline and a portion of fecal pellets was transferred to tissue-lysis buffer (MDT buffer) in a DNA tissue kit of Mini-80 system (Kurabo, Osaka, Japan). Fecal pellet was disrupted with beads beating. After proteinase K treatment, bacterial DNA was purified using Mini-80 system. Resultant DNA solution was evaluated for its DNA concentration and purity by Nano-drop ND-1000 spectrometer (Thermo Scientific) and Qubit 2.0 (Invitrogen) with Qubit™ dsDNA HS Assay Kit (Invitrogen). DNA was sent to BGI Japan, where shotgun library construction with KAPA HTP Library Preparation Kits (KAPA Biosystems) and paired-end sequencing was performed with the Illumina Hiseq X-Ten platform with the reagent Kit v2.5 (2 x 151 cycles). DNA sequence reads were assembled after elimination of low quality reads as previously (Tsuchida *et al.*, 2017). Obtained fasta files were subjected to antimicrobial resistant gene search using DIAMOND v0.9.10 against the Comprehensive Antibiotic Resistance Database (CARD) database (Buchfink *et al.*, 2015; McArthur *et al.*,

2013). The metagenomic data set was also examined by principal component analysis and heat map using statistical software R. The sequences have been deposited in the European Molecular Biology Laboratory (EMBL) under the accession number PRJEB20456.

Ethics statement

The protocol of this study was approved by the Uganda National Council of Science and Technology (UNCST), permit number A_522. A material transfer agreement was endorsed between College of Veterinary Medicine, Animal Resources, and Biosecurity, Makerere University and Kyoto Prefectural University for the materials included in the analysis. The material transfer was approved by UNCST. All the samplings were performed with a non-invasive manner in the presence of the farmers and district veterinary officers with their permission.

Results

Usage of antimicrobial agents

Our study sampled 13 farms: seven in Wakiso and six in Mpigi district. At each farm, we recorded the types of antimicrobial agents used (Table 1). In both districts, 53.8% (7/13) of farms used penicillins (mainly benzylpenicillin), 69.2% (9/13) used tetracyclines (mainly oxytetracycline), and 61.5% (8/13) used sulfonamides (with or without trimethoprim). Dihydrostreptomycin was the most frequently used aminoglycoside and often used in combination with benzylpenicillin. Macrolides and fluoroquinolones were not commonly used. Use of cephalosporins, carbapenems, old quinolones, colistin, and phenicols were not reported by the farmers. Penicillins (with or without aminoglycosides) were most commonly

used as an injection or spray for wound treatment for cattle and pigs. Tetracyclines were used as an injection for wounds or mastitis in cows and infection control and promotion of egg production in layers as feed or water additives. Sulfonamides (with or without trimethoprim) were used to treat diarrhea in cows and coccidiosis in chickens, diarrhea in cows and coccidiosis in chickens. Other drugs such as ivermectin, amprolium, albendazole, buparvaquone, and vitamins were used occasionally (data not shown).

Sample collection and isolation

After the isolation on ES Colimark agar media, 340 isolates were obtained. Among them, 293 isolates were positive for *E. coli*-specific PCR. Antimicrobial susceptibility test showed that 159 isolates were resistant to at least one antimicrobial agent. Twenty-five isolates which were 1) derived from the same feces, 2) showed the same antimicrobial resistance pattern, and 3) identified as the same phylogenetic type were considered as duplicated strains and omitted from further analyses. Overall, 134 unique *E. coli* strains (54 from Wakiso and 80 from Mpigi) were used for further analyses. Prevalence of each phylogenetic types among these 134 strains was as follows: A_0, n = 13; A_1, n = 56; B_1, n = 57; $B2_2$, n = 2; $B2_3$, n = 4; D_1, n = 1 and D_2, n = 1.

Antimicrobial resistance phenotypes and genotypes

Antimicrobial susceptibility tests revealed that TET-resistant isolates were very common in all livestock feces, with an overall prevalence of 97.0% (cattle 91.3%, pig 100%, goat 100%, layer 98.4%), followed by SXT resistance (56.7%) and AMP resistance (44.8%) (Table 2). A few isolates showed resistance to CFZ, KAN, MIN, NAL, CIP, CST, and CHL,

yet resistance to CTX and GEN were not detected. Isolates with co-resistance to AMP-TET-SXT were detected in all livestock, although the frequency was different (goat 62.5%, cattle 47.8%, layers 33.3%, and pig 15.0%). In addition, four isolates from layers showed resistance to six antimicrobial agents tested, including five different antimicrobial categories; two of them were resistant to AMP-KAN-TET-NAL-CIP-SXT, one was to AMP-KAN-TET-NAL-CHL-SXT, and one was to AMP-TET-NAL-CIP-CHL-SXT.

We analyzed the correlation between the frequency of resistance occurrence to penicillins (including AMP), tetracyclines, and sulfonamides in *E. coli* and the use of antimicrobial agents to livestock. The frequency of AMP-resistance in *E. coli* isolates from farms that used penicillins was significantly higher than that of *E. coli* from penicillin-free farms (odds ratio 2.03, 95% CI= 1.01 - 4.08, P =0.045 by chi-squared test) (Table 3).

PCR for antimicrobial resistance genes revealed that 83.3% (50/60) of AMP-resistant strains harbored bla_{TEM} as a beta-lactamase gene while bla_{SHV} and bla_{OXA} were not detected. Among TET-resistant strains (n = 130), 74.6% (97/130) harbored *tetA* gene, 9.2% (13/130) harbored *tetB* gene, 13.8% (18/130) harbored both *tetA* and *tetB*, and 0.8% (1/130) harbored *tetC* gene. Among SXT-resistant strains (n = 76), 11.8% (9/76) harbored *sul1* gene, 57.9% (44/76) harbored *sul2* gene, and 23.7% (18/76) harbored both *sul1* and *sul2*.

Metagenomic analysis

The reads for each predicted antimicrobial-resistant gene by CARD are shown in Figure 1. We classified them into 16 categories, according to the mode of antimicrobial-resistance. Among them, genes for multidrug efflux pumps were most prevalent in all except in one sample. In #972-3, a pig sample from a subsistence farm,

tetracycline-resistance genes were the most frequently identified while the frequency of multidrug efflux pump gene was lower than other samples (Fig 1). The representative genes of each category identified by CARD are summarized on Table 4, and the actual read percentages are listed on supporting information (Table S1). In total, we identified 911 antimicrobial-resistant genes. Multidrug efflux pump genes were the most frequent category (20.84% to 38.96% of total reads from each sample). Among them, *mdtB*, *mdtC*, and *mdtF*, genes which have been isolated from a plasmid in *Lactococcus lactis* subsp. *lactis* (Perreten *et al.*, 2001), were the three most representatives (2.90-3.72%, 2.33-3.65%, and 0.82-3.59%, respectively). The second most prevalent gene category was old/new quinolone resistance genes (9.83%-13.45%), the third was tetracycline resistance genes (4.78%-35.81%), and the fourth was polypeptides (including CST) resistance genes (5.58%-7.87%). Most resistant genes were encoded on bacterial chromosomal DNA such as bla_{ACT} for carbapenem resistance, *mfd* for quinolone resistance, and *arnA* for CST resistance. We also identified clinically important resistance genes which are often encoded on plasmidic DNA such as $bla_{\text{CTX-M}}$ for cephalosporin resistance, *qnr* for fluoroquinolone resistance, *ermB* for macrolide resistance, *vanA* and *vanB* for vancomycin resistance, and *vatA* for streptogramin resistance, while the frequencies of reads were not as high (Table S1). The heat mapping and principal component analysis using metagenomic read numbers showed that antimicrobial resistance genes identified from each sample were quite different (Figure S1).

Discussion

This study describes ARB in livestock in Uganda. There have been several reports on ARB in Ugandan livestock (Afema *et al.*, 2016; Byarugaba *et al*, 2011); however, these studies were focused on cultivation of ARB only. Our study describes the use of

antimicrobials on farms and clarifies ARB in livestock, as well as the distribution of antimicrobial-resistance genes in ARB.

In our study, inquiries to the farmers revealed that the usage of benzylpenicillin, oxytetracycline, and sulfonamides (with or without trimethoprim) was common on Ugandan livestock farms. Penicillins, with or without streptomycin, were often administered as an injection or spray to cattle and pigs, while oxytetracycline and sulfonamides were orally administered to layers through feeds or drinking water. In addition, antimicrobial susceptibility testing showed that resistance to AMP, TET, and SXT was common in the strains detected in our study among our strains, yet still co-resistance to these three antimicrobials was also detected. Previous studies on *E. coli* derived from Ugandan livestock have reported the same outcome (Afema *et al.*, 2016; Byarugaba *et al*, 2011). Such characteristics were identical to the common antimicrobial agents which have been used at the sampling sites. These results support the theory that the use of antimicrobial agents contributes to the prevalence of ARB by providing selective pressure (Asai *et al.*, 2005).

The dissemination of ESBL-producing *E. coli* and other *Enterobacteriaceae* in livestock has been reported globally (Carattoli, 2008). A previous study on ARB isolated from clinical specimens of human hospital in Uganda showed high frequency of cephalosporin-resistant *E. coli*; 77.8% were resistant to ceftazidime (a third-generation cephalosporin) and 69.4% were resistance to cefepime (a fourth-generation cephalosporin) (Seni *et al.*, 2013). In contrast, we didn't detect CTX (a third-generation cephalosporin) resistant *E. coli* from livestock in our study. According to our data, no farms used third-generation cephalosporins or carbapenems for the livestock. Although we have no data on the availability of those antimicrobial agents in Wakiso and Mpigi districts, we speculate that cephalosporins and carbapenems are too expensive for the farmers to treat their livestock.

Consequently, we considered the absence of CTX-resistant *E. coli* in livestock resulted from the lack of exposure of CTX to the gut microbiota in Ugandan livestock. It is also possible that the difference in prevalence of cephalosporin resistance in *E. coli* suggests that the emergence and spread of cephalosporin-resistant *E. coli* in Uganda is not through zoonotic transmission but through medical use. However, the results of metagenomic analysis in our study also identified $bla_{\text{CTX-M}}$-type ESBL genes and several carbapenem-resistance genes among fecal bacteria. Although we cultured *E. coli* only, this result indicates that $bla_{\text{CTX-M}}$ and carbapenem-resistance genes were not harbored by *E. coli* but harbored by other fecal bacteria, suggesting the possibility of horizontally transfer of those resistance genes from fecal bacterial population to *E. coli*. Previous studies suggested that the emergence of ARB is associated with the use of antimicrobial agents (Asai *et al.*, 2005, Sato *et al.*, 2014). Therefore, the emergence of cephalosporin and/or carbapenem resistant *E. coli* may occur if these antimicrobial agents are used indiscriminately on Ugandan farms. As the dissemination of ESBL- or carbapenemase-producing *Enterobacteriaceae* has become a global concern, the use of these antimicrobials and the presence of ARB in Uganda should be monitored carefully.

We detected several *E. coli* strains that showed resistance to CHL and/or CST. However, phenicols (such as CHL and thiamphenicol) and CST were not used on the farms we visited. Although we did not investigate the availability of phenicols and CST in Wakiso and Mpigi districts, both antimicrobial agents are inexpensive compared with cephalosporins or carbapenems, and have been frequently used for livestock in other countries. In our study, antimicrobial use was self-reported by the farmers and determined by the researchers based on the drug bottles found at each farm. Therefore, there were some limitations that the results of interview may not be accurate representation of antimicrobial use on the farms. In addition, there is a possibility that CHL- and/or CST-resistant strains have been transmitted

from the farmers or livestock on farms in the surrounding area, which have used CHL and/or CST. Metagenomic analysis revealed that resistance genes for phenicols (CHL) and polypeptides (CST) were detected in the fecal samples. This suggest that, as same as $bla_{\text{CTX-M}}$ and carbapenemase genes, CHL- and CST-resistant genes were already spread in fecal bacterial population of Ugandan livestock.

When comparing the result between animal species, multidrug-resistant strains that showed resistance to five different categories of antimicrobial agents were only identified from layers. Compared with other livestock, layers are frequently administered antimicrobial agents orally, as a food or water addictive, not only for the treatment of bacterial diseases but also as growth promoter (Brown *et al.*, 2017). Consequently, the presence of multidrug-resistant *E. coli* in layers may be the result of the frequent or indiscriminate use of antimicrobial agents. These multidrug-resistant strains in layers are problematic for farmers because colibacillosis is one of major problem in layer production and causes huge economic losses (Bengtsson and Greko, 2014; Dziva and Stevens, 2008).

The results of the metagenomic analysis revealed that the frequency of ARB in the subsistent farm sample (#927-3) was different from other samples from commercial farms. In the sample, the frequency of multidrug efflux pump gene was lower than others. According to the owner of the subsistence farm, no antimicrobial agents were used at that time on the farm. We considered that the absence of antimicrobial use in the farm is the reason of low frequency of multidrug efflux pump gene in the sample. In contrast, the frequency of tetracycline resistance gene in the subsistent farm sample was much higher than others. According to the CARD analysis data, the main tetracycline-resistance gene in the subsistence farm sample is *tetW* (Table S1). In a previous study, the prevalence of *tetW* is reported to be clearly related to the usage of tetracycline (Tsuchida *et al.*, 2017). The reason

why *tetW* gene was frequently detected from the subsistence farms sample is unclear. One possible explanation for this result is that the owner of the farm had used tetracycline before, and *tetW* gene had remained on the farm. A previous study reported that tet genes including *tetW* can remain without selection pressure (Tamminen *et al.*, 2011). We need more surveillance to clarify the difference in the frequency of antimicrobial resistance genes between commercial farms and subsistence farms because the sample number of subsistence farm in this study was only one.

Metagenomic analysis also detected antimicrobial-resistance genes for Gram-positive bacteria, such as macrolide resistance and vancomycin resistance genes (*erm* and *van* variants, respectively). This suggests that the presence of Gram-positive ARB was also common in the fecal samples we studied. As a previous Ugandan study have reported the presence of antimicrobial-resistant enterococci (Byarugaba *et al*, 2011), further genetic study on *Enterococcus* spp. in Ugandan livestock is required.

A limitation of this study is the criteria we used to determine duplicated *E. coli* strains. We determined duplicated strains by the origin, antimicrobial resistance phenotype, and phylogenetic type of the strains only. We excluded 25 duplicated isolates from analyses, but it might contain genetically distinct strains. Although we believe that the main phenotypic and genotypic features of antimicrobial resistance of *E. coli* in Ugandan livestock were clarified by our present data, other methods to check the duplication of strains such as repetitive element palindromic PCR or random amplified polymorphic DNA analysis should be performed.

In conclusion, we identified ARB in Ugandan livestock using both the culture-dependent method (isolation of antimicrobial-resistant *E. coli*) and culture-independent method (metagenomic analysis on antimicrobial-resistant genes) for the

first time. Reports on ARB among animals in Africa is increasing, but there are still limited data available. Our results provide essential information on the prevalence of ARB in livestock in Uganda. Although we could not isolate third-generation cephalosporin-resistant *E. coli*, there is a risk of the emergence of cephalosporin- or carbapenem-resistant *Enterobacteriaceae* in Ugandan livestock because the genes for ESBLs and carbapenemases have already exist in their gut microbiota.

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Conflict of interest statement

The authors declared no conflict of interest. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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Table 1. Summary of the information of farms, fecal samples and E. coli strains in this study.

	Wakiso district	Mpigi district	Total
Number of farms	7	6	13
Use of antimicrobial agents			
Penicillins	42.9% (3/7)	66.7% (4/6)	53.8% (7/13)
Cephalosporins	0	0	0
Carbapenems	0	0	0
Aminoglycosides	42.9% (3/7)	33.3% (2/6)	38.5% (5/13)
penicillin-streptomycin combination	28.6% (2/7)	33.3% (2/6)	30.8% (4/13)
Tetracyclines	57.1% (4/7)	83.3% (5/6)	69.2% (9/13)
Macrolides	14.3% (1/7)	16.7% (1/6)	15.4% (2/13)
Old quinolones	0	0	0
Fluoroquinolones	14.3% (1/7)	16.7% (1/6)	15.4% (2/13)
Colistin	0	0	0
Phenicols	0	0	0
Sulfonamides	57.1% (4/7)	66.7% (4/6)	61.5% (8/13)
sulfonamides-trimethoprim combination	28.6% (2/7)	33.3% (2/6)	30.8% (4/13)
Fecal samples			
Cattle	15	30	45
Pig	20	10	30
Goat	0	16	16
Layer	14	25	39

Total	49	81	130
***E. coli* strains used in this study**			
Cattle	5	18	23
Pig	29	11	40
Goat	0	8	8
Layer	20	43	63
Total	54	80	134

Table 2. Frequency of antimicrobial resistance of E. coli isolates in this study.

	Cattle (n = 23)†	Pig (n = 40)	Goat (n = 8)	Layer (n = 63)	Total (n = 134)
AMP	18 (78.3%)	10 (25.0%)	6 (75.0%)	26 (41.3%)	60 (44.8%)
CFZ	2 (8.7%)	2 (5.0%)	0	2 (3.2%)	6 (4.8%)
CTX	0	0	0	0	0
GEN	0	0	0	0	0
KAN	0	0	0	5 (7.9%)	5 (3.7%)
TET	21 (91.3%)	39 (100%)	8 (100%)	62 (98.4%)	130 (97.0%)
MIN	0	8 (20.0%)	0	1 (1.6%)	9 (6.7%)
NAL	4 (17.4%)	5 (12.5%)	1 (12.5%)	19 (30.2%)	29 (21.6%)
CIP	1 (4.3%)	0	0	10 (15.9%)	11 (8.2%)
CST	2 (8.7%)	0	0	0	2 (1.5%)
CHL	1 (4.3%)	3 (7.5%)	0	5 (7.9%)	9 (6.7%)
SXT	13 (56.5%)	13 (32.5%)	7 (87.5%)	43 (68.3%)	76 (56.7%)
AMP+TET+SXT	11 (47.8%)	6 (15.0%)	5 (62.5%)	21 (33.3%)	43 (32.1%)
Five different categories‡	0	0	0	4 (6.3%)	4 (3.0%)

Abbreviations: AMP, ampicillin; CFZ, cefazolin; CTX, cefotaxime; GEN, gentamicin; KAN, kanamycin; TET, tetracycline; MIN, minocycline; NAL, nalidixic acid; CIP, ciprofloxacin; CST, colistin; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim.

† The number of fecal samples derived from each livestock species.

‡ Among four strains which were resistance to 5 different categories of antimicrobials, two of them were resistant to AMP-KAN-TET-NAL-CIP-SXT, one to AMP-KAN-TET-NAL-CHL-SXT and one to AMP-TET-NAL-CIP-CHL-SXT.

Table 3. Frequency of antimicrobial resistance and correlation with the use of antimicrobial agents in livestock.

	Strains from farms with usage of respective antimicrobial	Strains from farms without usage of respective antimicrobial	Odds ratio (95% CI)	P value†
Penicillin resistance	28.4%	16.4%	2.03 (1.01 - 4.08)	0.045
Tetracycline resistance	83.6%	13.4%	6.22 (0.29 - 16.71)	0.095
Sulfonamide resistance	42.5%	14.2%	1.14 (0.49 - 2.30)	0.735

Abbreviation: CI, confidence interval.

† P value was calculated using chi-squared test for penicillin and sulfonamide resistance and Fisher's exact test for tetracycline resistance.

Table 4. Antimicrobial resistance genes identified by metagenomic analysis.

Resistance gene (accession no.)	#927-4 Cattle commercial	#927-8 Pig commercial	#927-10 Pig commercial	#927-3 Pig subsistence	#927-11 Layer commercial	#927-12 Layer commercial
(1) Beta-lactams	**2.39% (2163)‡**	**0.80% (2471)**	**0.87% (2059)**	**1.11% (658)**	**0.64% (2413)**	**0.52% (1561)**
bla$_{MIR-15}$ (AIT76104.1)†	0.07% (63)	0.06% (198)	0.07% (155)	0.02% (14)	0.09% (352)	0.07% (219)
bla$_{TEM-87}$ (AAG44570.1)	0.24% (222)	0.01% (21)	0.04% (100)	0.01% (4)	0.01% (24)	0.03% (27)
bla$_{CFE-1}$ (BAC76072.1)	0.03% (29)	0.04% (110)	0.03% (62)	0.01% (6)	0.03% (110)	0.02% (71)
(2) Carbapenems	**0.14% (129)**	**0.08% (249)**	**0.09% (217)**	**0.12% (73)**	**0.13% (494)**	**0.40% (1188)**
bla$_{ACT-1}$ (AAC45086.2)	0.04% (39)	0.03% (100)	0.03% (82)	0.01% (4)	0.05% (192)	0.04% (110)
bla$_{ACT-12}$ (AFU25650.1)	0.04% (35)	0.02% (62)	0.02% (48)	0.01% (4)	0.03% (120)	0.03% (95)
bla$_{ACT-28}$ (AHL39333.1)	0.04% (33)	0.02% (49)	0.02% (45)	0.01% (6)	0.04% (139)	0.02% (65)
(3) Aminoglycosides	**0.50% (451)**	**7.63% (23671)**	**7.23% (17212)**	**2.11% (1253)**	**4.47% (16859)**	**6.09% (18125)**
aph(3")-Ib (ABK33456.1)	0.14% (126)	3.54% (10980)	3.17% (7550)	0.47% (280)	1.51% (5700)	1.87% (5573)
aph(6)-Id (AAC23556.1)	0.14% (125)	3.48% (10827)	3.22% (7669)	0.39% (231)	1.34% (5030)	1.88% (5606)
ant(6)-Ib (AIJ27543.1)	0.06% (53)	0.22% (667)	0.29% (693)	0.71% (422)	0.73% (2756)	0.28% (821)
(4) Old/New quinolones	**13.45% (12186)**	**13.02% (40380)**	**11.77% (28023)**	**9.83% (5843)**	**12.93% (48708)**	**13.31% (39624)**
mfd (NP_415632.1))	3.11% (2821)	3.95% (12255)	3.59% (8537)	3.17% (1885)	3.07% (11551)	3.49% (10393)
emrB (BAA16547.1)	1.48% (1338)	1.85% (5739)	1.68% (4005)	1.54% (917)	1.80% (6764)	1.80% (5366)
patA (NP_417544.5)	1.51% (1369)	1.66% (5162)	1.55% (3679)	0.71% (422)	1.63% (6133)	1.67% (4978)
(5) Phenicols	**2.49% (2255)**	**0.01% (16)**	**0.34% (810)**	**0.32% (190)**	**0.03% (97)**	**0.05% (148)**
catII (CAA37805.1)	2.43% (2203)	0.01% (12)	0.06% (136)	0.24% (142)	0.01% (18)	0.01% (9)
cat (AAL08441.1)	< 0.01% (2)	< 0.01% (2)	0.14% (326)	0	< 0.01% (3)	0
catI (CAA23899.1)	0.02% (2)	0	0.12% (276)	0	0	0
(6) Fosfomycin	**0.01% (7)**	**0.07% (220)**	**0.01% (18)**	**0.06% (34)**	**0.02% (68)**	**0.11% (341)**
fosA2 (ACC85616.1)	< 0.01% (3)	< 0.01% (5)	< 0.01% (4)	0.03% (19)	0	0.10% (298)

fosA5 (AJE60855.1)	0	0.06% (192)	< 0.01% (1)	0.01% (4)	<0.01% (2)	0
murA (CCE36834)	< 0.01% (4)	< 0.01% (9)	0.01% (12)	0.01% (5)	0.01% (46)	0.01% (32)
(7) Lincosamides	**0.35% (318)**	**0.03% (89)**	**0.04% (96)**	**0.14% (86)**	**0.08% (310)**	**0.07% (210)**
lmrD (ABF66027.1)	< 0.01% (1)	< 0.01% (14)	0.01% (24)	0.01% (3)	0.07% (253)	0.03% (52)
lnuC (AAY32951.1)	0.13% (120)	0.02% (49)	0.03% (60)	0.10% (57)	0	< 0.01% (6)
lsaB (NP_899166.1)	0.21% (189)	< 0.01% (14)	< 0.01% (3)	0.03% (17)	< 0.01% (9)	< 0.01% (12)
(8) Macrolides	**0.24% (217)**	**0.26% (807)**	**0.44% (1048)**	**1.85% (1099)**	**1.41% (5329)**	**0.94% (2793)**
mefA (YP008997285.1)	0.07% (63)	0.08% (262)	0.32% (750)	1.30% (771)	0.10% (388)	0.06% (178)
ermB (AAC45607.1)	0.01% (12)	0.01% (27)	0.01% (26)	0.03% (16)	0.27% (1034)	0.13% (3989)
ermQ (AAC36915.1)	< 0.01% (2)	< 0.01% (12)	< 0.01% (10)	0.05% (37)	0.14% (516)	0.15% (461)
(9) Tetracyclines	**10.93% (9988)**	**6.68% (20702)**	**7.61% (18104)**	**35.81% (21280)**	**6.38% (24041)**	**4.78% (14230)**
tetW (ACA23185.1)	2.65% (2406)	2.90% (8987)	3.45% (8221)	18.30% (10871)	2.83% (10669)	1.18% (3518)
tetC (AAO16462.1)	1.22% (1109)	1.14% (3522)	0.96% (2295)	0.16% (94)	0.91% (3447)	0.55% (1646)
tet40 (AFK31666.1)	2.07% (1875)	0.56% (1744)	0.75% (1784)	5.57% (3309)	0.04% (140)	0.08% (232)

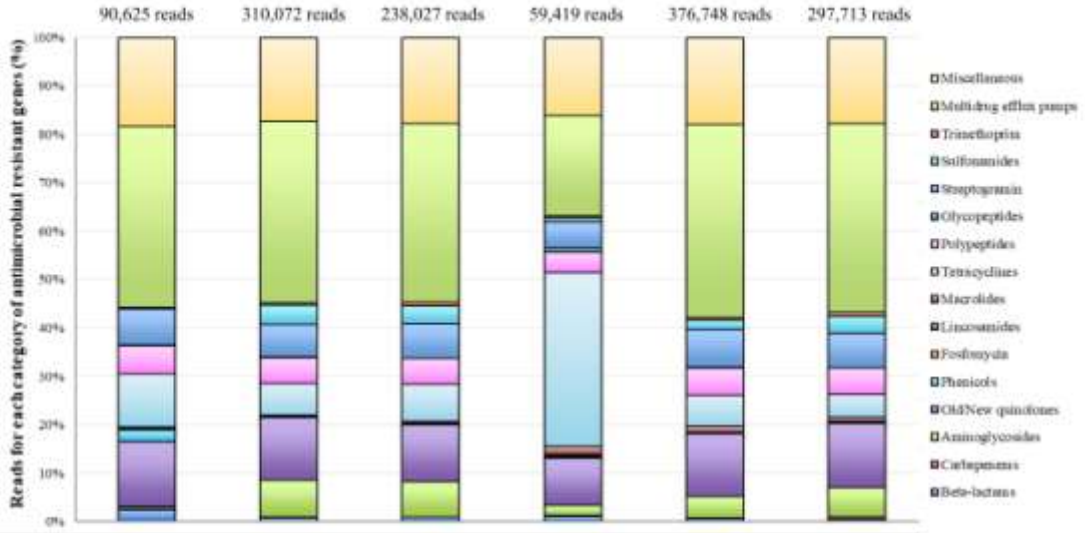
Table 4. (continued)

Resistance gene (accession no.)	#927-4 Cattle commercial	#927-8 Pig commercial	#927-10 Pig commercial	#927-3 Pig subsistence	#927-11 Layer commercial	#927-12 Layer commercial
(10) Polypeptides	**7.46% (6761)**	**6.80% (21085)**	**7.14% (16985)**	**5.58% (3313)**	**7.87% (29436)**	**7.18% (21378)**
arnA (AAC75315.1)	1.64% (1487)	1.43% (4439)	1.84% (4390)	1.12% (663)	2.15% (8110)	1.76% (5232)
pmcC (BAE78116.1)	1.56% (1411)	1.42% (4405)	1.30% (3103)	0.44% (260)	1.69% (6350)	1.32% (3943)
pmrB (YP_492255.1)	1.24% (1122)	1.02% (3162)	1.03% (2445)	0.35% (207)	1.24% (4690)	1.00% (2990)
(11) Glycopeptides	**0.23% (208)**	**0.03% (81)**	**0.03% (69)**	**0.68% (406)**	**0.01% (32)**	**0.02% (58)**
vanRG (ABA71727.1)	0.03% (29)	< 0.01% (10)	< 0.01% (8)	0.14% (82)	< 0.01% (7)	< 0.01% (12)
vanYG1 (ABA71729.1)	0.02% (17)	< 0.01% (9)	0.01% (13)	0.12% (71)	< 0.01% (6)	< 0.01% (4)
vanRI (WP_011461303)	0.02% (17)	< 0.01% (7)	0.01% (17)	0.09% (55)	< 0.01% (3)	< 0.01% (5)
(12) Streptogramin	**0.04% (147)**	**0.02% (12279)**	**0.04% (8841)**	**0.11% (414)**	**0.23% (7529)**	**0.09% (281)**
vgaC (CBY88983.1)	0.01% (7)	0.02% (60)	0.03% (61)	0.03% (19)	0.23% (792)	0.08% (248)
vatB (AAA86871.1)	0.02% (17)	< 0.01% (6)	< 0.01% (3)	0.08% (45)	0.01% (21)	< 0.01% (5)
vatE (AAF86220.1)	0.01% (5)	0	< 0.01% (1)	< 0.01% (2)	0.01% (32)	< 0.01% (5)
(13) Sulfonamides	**0.16% (147)**	**3.96% (12279)**	**3.71% (8841)**	**0.70% (414)**	**2.00% (7529)**	**3.59% (10693)**
sul2 (AAL59753.1)	0.12% (109)	3.86% (11977)	3.42% (8147)	0.47% (279)	1.30% (4884)	1.81% (5392)
sul1 (AEJ33969.1)	0.04% (32)	0.09% (283)	0.19% (450)	0.22% (2499)	0.66% (4999)	1.68% (8392)
sul2 (WP_063855569)	0	0.01% (16)	0.10% (229)	0.01% (4)	0.03% (97)	0.03% (91)
(14) Trimethoprim	**0.11% (99)**	**0.40% (1231)**	**0.79% (1887)**	**0.43% (253)**	**0.37% (1399)**	**0.75% (2220)**
dfrA14 (ACI32877.1)	0.03% (24)	0.33% (1021)	0.67% (1584)	0.12% (69)	0.08% (305)	0.25% (752)
dfrA1 (YP_001715373.1)	0.01% (8)	0.01% (24)	0.04% (91)	0.08% (49)	0.16% (599)	0.20% (602)
dfrE (AAD01868.1)	0.05% (45)	0.04% (124)	0.048% (89)	0.18% (109)	0.10% (359)	0.07% (217)
(15) Multidrug efflux pumps	**37.39% (33884)**	**37.61% (116621)**	**36.90% (87836)**	**20.84% (12383)**	**40.05% (150905)**	**38.96% (3115976)**

mdtB (AAC75136.1)	2.90% (2624)	3.72% (11527)	3.35% (7981)	2.46% (1462)	3.18% (11995)	3.56% (10592)
mdtC (AAC75137.1)	2.73% (2474)	3.65% (11326)	3.34% (7947)	2.33% (1387)	3.04% (11446)	3.34% (9946)
mdtF (AAC76539.1)	3.18% (2878)	2.42% (7502)	3.27% (7776)	0.82% (489)	3.59% (13528)	2.94% (8756)
(16) Miscellaneous	**24.12% (21860)**	**22.61% (70103)**	**22.99% (54730)**	**20.31% (12068)**	**23.38% (88073)**	**23.14% (68887)**
Total	**100% (90625)**	**100% (310072)**	**100% (238027)**	**100% (59419)**	**100% (376748)**	**100% (297713)**

† Representative genes of top three read percentage in each category are listed.

‡ Numbers in the brackets were the read numbers of each gene.



Code	#927-4	#927-8	#927-10	#927-3	#927-11	#927-12
Farm name	Wakiso - 1	Wakiso - 2		Wakiso - 3	Wakiso - 4	
Livestock	Cattle	Pig		Pig	Layer	
Farm style	Commercial	Commercial		Subsistence	Commercial	
Antimicrobial usage†	P, T, S	P, T		none	T, S	
Frequency of antimicrobial-resistant *E.coli* in the farm‡	AMP: 1/1 TET: 1/1 SXT: 1/1	AMP: 3/15 TET: 15/15 SXT: 5/15		AMP: 2/2 TET: 2/2 SXT: 2/2	AMP: 3/7 TET: 7/7 SXT: 6/7	