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Metagenomic analysis of enteric bacterial pathogens affecting the performance of dairy cows in smallholder productions systems

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There is little information about the diversity of bacterial pathogens present in the rumen and feces of healthy cow and the subsequent effects on the performance of the host animal. The objectives of the present study were to genetically characterize the enteric bacterial pathogens found in the rumen fluid and cow feces and to identify the resistant genes responsible for antimicrobial resistance in the detected pathogens. The cow feces and rumen fluid samples (6 rumen fluid and 42 feces) were collected from lactating dairy cows. Using next generation sequencing, the enteric bacterial pathogens detected were screened for antimicrobial resistance genes using ResFinder-2.1 database in the center of Abricate. The characterized enteric bacterial pathogens include *Escherichia coli, Salmonella enterica, Streptococcus agalactiae, Streptococcus pyogenes, Campylobacter coli,* and *Campylobacter fetus* among others. Those enteric bacterial pathogens were also drug resistant bacteria except *Campylobacter coli*. The *Campylobacter fetus fetus* was identified as the only multidrug resistant bacterial pathogen detected in the cow feces. However, the abundant resistant genes detected confer resistance to tetracycline (17 genes from 209 contigs), beta-lactam (21 genes from 67 contigs), streptomycin (6 genes from 153 contigs), and sulfamethoxazole (2 genes from 72 contigs). This is the first study to identify the diversity of enteric bacterial pathogens from the station based and smallholder dairy cows in Kenya and Tanzania, respectively.

Key words: Antimicrobial resistant genes, enteric bacterial pathogens, dairy cows, next generation sequencing.

INTRODUCTION

Bovine ruminant particularly dairy cow contributes to the nutrition and wellbeing of humans' world widely by providing a variety of dairy products such as milk and its derivatives (Zhu, 2016). Milk production is of great economic concern to farmers and the quality is closely associated with the health of human beings.

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Improvement of its production in ruminant research relies on our understanding of the “microbial organ” of dairy cows (Petri et al., 2013; Zhu, 2016). Dairy cows have evolved a symbiotic relationship with a complex microbiome consisting of bacteria, fungi, and protozoa located in the reticulo-rumen that breakdown ingested food (Ross et al., 2012; Creevey et al., 2014; Pitta et al., 2016). These gut microbiome confer metabolic and immunological benefits to the host animal (Peng et al., 2015).

The rumen microbiota adapts rapidly to the intervention methods such as dietary formulations, biological feed additives and chemo-genomics of the host animal (Roehe et al., 2016). Therefore, nutrition represents an important tool for manipulating the microbial ecosystem to optimize rumen function while producing high-quality meat and milk for human consumption (Loor et al., 2016). However, the molecular characterization of the rumen microbiota is a viable option with regards to their effects on performance of dairy cows. Moreover, those animals produce large quantities of manure with a wide variety of pathogenic or non-pathogenic microorganisms to the dairy cows (Manyi-Loh et al., 2016). The bacterial pathogens found in the rumen nutrients are absorbed in the blood system and cause reproductive tracts infections such as mastitis and retained placenta (Wang et al., 2013) that affects the dairy industry (Wang et al., 2013). However, the techniques for identifying unidentified bacterial pathogens depend on the specific requirements for the species in the laboratories. The routine diagnostic techniques use different culturing methods, media, and reagents (Nakamura et al., 2008). Various bacteria require specific growth conditions and fail to grow in a given culture medium which leads to difficulties in handling samples in clinical microbiology laboratories (Castillo et al., 2006).

Furthermore, the feces shed from the gastro-intestinal tract (GIT) of the dairy cow are used in the agricultural farming. However, it presents the main source of antimicrobial resistance genes to the smallholder farms and the host animal (Zhang et al., 2015). The resistance genes are horizontally transferred in the bacterial species in the gut before fecal excretion (Chambers et al., 2015). Resistant genes excreted from animal gut contaminate the farms and may reach the human population through the consumption of dairy products. The use of the antimicrobials especially at sub therapeutic levels in the dairy farms selects for antimicrobial resistant (AMR) bacteria which contain the AMR genes (Sawant et al., 2007; Akindolire et al., 2015; Cameron and McAllister, 2016). The antimicrobial resistant pathogens contribute to increased mortality and morbidity of dairy cattle in the cattle production system which causes significant losses to dairy farmers (Call et al., 2008). The identification and characterization of the resistance genes present in the gut microbiome of the host animal was previously performed with routine diagnostic techniques such as the conventional culture methods, and were found to be insufficient and informative (Sawant et al., 2007).

Moreover, we depend on antimicrobials for the treatment of dairy cattle affected with pathogenic microorganisms (Idriss et al., 2014). The indiscriminate use of these antimicrobials in dairy cows benefits the development of resistant strains in the host animals (Idriss et al., 2014). However, little information is known about the source, diversity and distribution of antimicrobial resistance genes in the most non-culturable environmental bacterial pathogens (Chambers et al., 2015). Therefore, the use of bioinformatic approaches together with high-throughput sequencing techniques (HTS) in the analyses of microbiome overcome various methods used in the characterization of environmental microbes (Jami et al., 2014). Additionally, the use of next generation sequencing (NGS) techniques, bioinformatic tools, and molecular based approaches is the potential laboratory way for studying the diversity of rumen microbiota of health individuals (Dowd et al., 2008). The HTS techniques (Illumina sequencing and Roche/454 pyrosequencing) are mainly used in the detection and characterization of diversity of microbiomes by analyzing 16S rRNA gene amplicons in the current decade (Klein-Jöbstl et al., 2014). However, the analyses of data obtained from these sequencing technologies need advanced computational approaches and powerful machines (Jovel et al., 2016). These cause problems for microbiologists and laboratory clinicians when studying the diversity of microbiome (Jovel et al., 2016).

In this regards, metagenomics, the genomic analysis of population of microorganisms makes possible the profiling of environmental microbiome (Bashir et al., 2014; Flygare et al., 2016). Metagenomics allows the identification and characterization of the composition of microbiota as well as the abundance of their genes (Roehe et al., 2016). However, this platform provide an important advantage as the single DNA fragments of a library is sequenced directly without cloning and compares the sequences to known sequence database (Barzon et al., 2011; Nathani et al., 2013). Therefore, the objectives of the present study were to identify and genetically characterize the enteric bacterial pathogens found in the cow feces and rumen fluid of dairy cows affecting their performance using metagenomic approaches. In addition, it also identified the genes responsible for antimicrobial resistance in the detected bacterial pathogen.

MATERIALS AND METHODS

Ethics statement

The study was approved by the institutional ethics committee of the University of Nairobi (UoN), Faculty of Veterinary Medicine guidelines and the International Livestock Research Institute (ILRI)-Institutional Animal Care and Use Committee (IACUC). The study was conducted in accordance to the good scientific practices

approved by the two institutions. The animals were restrained by the experienced veterinary professionals during the data collection to reduce the discomfort.

Study sites

This study was carried out in one site in Kenya (University of Nairobi (UoN) Faculty of Veterinary Medicine farm) and two study sites in Tanzania namely; Lushoto and Rungwe. The UoN Faculty of Veterinary Medicine farm is located on a 375 acre piece of land in Kanyariri Village of Kiambu County in Kenya at latitude 1°14'33.4"S and longitude 36°42'36.3"E (https://www.uonbi.cavs.ac.ke). Lushoto district is located in Tanga region which lies at latitudes 4° and 6°S and longitudes 38° to 39°E (Mfune, 2015). Rungwe district lies between latitudes 9° 00 and 9° 30 E and longitudes 33 °E and 34°S in Mbeya region (Karwani et al., 2016).

Animals and experimental treatments

Six adults lactating dairy cows reared at the UoN Faculty of Veterinary Medicine farm were used in the experiment. The criteria for selection were based on the breed, body condition, medical history and cows in their first stage of lactation. Two lactating dairy cows were selected in each genotype namely Jersey, Friesian and Jersey×Friesian cross. The animals had an average body weight ± standard deviation of 300 ± 50 kg and were 174 ± 15 days into their first lactation. The experimental animals were assigned to a completely randomized design (CRD) experiment with a 3 × 3 factorial arrangement of treatments. The treatments were three cattle genotypes (Friesian (Fri), Jersey (Jer) and Friesian X Jersey cross (CB)) and three diets (90% crop residue and 10% concentrate, followed by 75% crop residue and 25% concentrate and then 60% crop residue and 40% concentrate). The three rations were formulated to meet the energy requirements of cows yielding 20 kg of milk/day with 4.0% milk fat and 3.5% true protein, by the NRC - Nutrient Requirements of Dairy Cattle Software v 1.9 (NRC, 2001). Feeds were offered *ad libitum* as a total mixed ration (TMR) to avoid the selection of dietary components. The dietary components for the crop residue were: Rhodes grass (*Chloris gayana*) ray, rapier grass (*Pennisetum purpureum*), kikuyu grass (*Pennisetum clandestinum*), maize (*Zea mays*) stover. These were mixed with dairy meal and urea at different proportions to make the three diets. The chemical composition of the dietary components was assayed according to the Association of Official Analytic Chemist (AOAC) methods (1998) while the dietary fiber determination was conducted according to Van Soest et al. (1991).

After a 10-day acclimatization period, the cattle were fed the three different diets in three consecutive 10-day periods. Experimental diets were offered in two meals at 8am and 6pm, one-half of the allowed daily rations at each feeding. Throughout the 30 days of the experiment, the cattle were housed in stalls and given free access to fresh water and mineral supplement.

Sample collection

Two sample types (fecal grab and rumen fluid) were collected from each experimental animal. Rumen samples were collected via a flexible stomach tube while fecal grabs were collected from the rectum. Furthermore, thirty six (36) fecal samples were collected from selected animals in Lushoto and Rungwe districts in Tanzania. Approximately, 250 to 500 g of individual fecal samples were collected from the rectum of each cattle using a clean palpation sleeve and sterile lubricant for each collection and a sub-sample transferred into sterile 50 ml falcon tube (Chambers et al., 2015). In total, four serial samples (for each sample type) were collected from each of the experimental animals during the feeding experiment. The samples were collected at days 0, 10, 20 and 30 (that is, on the last day of the 10 days on each experimental feed). Samples were collected approximately 2 h after the morning feeding. The samples were immediately kept on ice in the cool box and shipped to the Biosciences east and central Africa (BecA-ILRI) Hub, laboratory at the International Livestock Research Institute where they were stored at -20ºC until microbial DNA analysis. Frozen samples were thawed at room temperature before being mixed thoroughly by vortexing for 30 s at maximum speed.

DNA extraction and library construction with Illumina sequencing

Total genomic DNA was extracted from all samples using the commercially available QIAamp DNA Stool Mini Kit (Qiagen, USA) according to the manufacturer's instructions but with a few modifications. The modifications included: (i) Using double the recommended sample volume and (ii) addition of 2 µl of RNAse A after mixing propeinase K and the sample. The DNA concentration and quality were assessed by Nanodrop spectrophotometry (Nanodrop Technologies), Qubit® 2.0 Fluorometer with the Qubit ®dsDNA HS Assay Kit and agarose gel electrophoresis (Onate et al., 2015). The recovered DNA products were stored at -20°C until further analysis. Next, the Nextera XT DNA library preparations was performed following the workflow and protocol described by Kim et al. (2013) followed by Illumina Miseq Sequencing. Briefly, 50 ng of genomic DNA were first tagmented in a transposase-mediated reaction that simultaneously fragments and tags DNA with adapters. The adapter-tagged DNA fragment libraries were purified with Zymo Kit to remove unwanted constituents from the tagmentation reaction. Subsequently, the sequencing adapters were added to the fragment library by limited-cycle PCR, and finally the DNA was size-selected for sequencing and finally paired end sequencing was performed using the Illumina MiSeq v3 (Illumina) System.

Quality control of the raw sequence reads and K-mer analysis

In this study, 48 samples (12 were collected at the UoN Faculty of Veterinary Medicine farm and 36 from the smallholder farms in Tanzania) were used. The quality of the data was checked using fastQC/v0.11.2. Then, Sickle/v1.33 was used for trimming of the low quality reads at the length threshold of 100 bps and the quality threshold of 20 (Q=20). Thereafter, K-mer analysis of these raw NGS sequence reads was determined prior to filtering and functional annotation of the reads (Onate et al., 2015). Kmergenie/v1.7044 (Sievers et al., 2017), an efficient single program written in C/C++, was used for this process. The frequencies of different k-mer abundance value contained in a set of reads were plotted as a k-mer abundance histogram (Chikhi and Medvedev, 2013; Onate et al., 2015). Finally, the optimal k value that maximizes the number of genomic k-mers (Chikhi and Medvedev, 2013) was k=23 (optimal k-mer) was identified.

Metagenomic assemblies of the reads and taxonomic annotation of the contigs

The *De novo* assembly of the quality filtered reads after trimming, was performed using the Ray/v2.3.1 to give the larger fragments technically known as contigs and scaffolds in fasta files. The functional annotation of the contigs was performed using Prokka homology-search against the protein reference in the Diamond database. However, the taxonomic annotation and analysis was

done using the CAT (Contig Annotation Tool) pipeline utilizing the rapid prokaryotic genome annotation (Prokka) described in details by Seemann (2014).

Taxonomic characterization and detection of enteric bacterial pathogens

The taxonomic visualization of the bacterial species present in the metagenome was performed using Krona tool/v 2.7. The classified contigs representing the cow rumen fluid and feces at the UoN Faculty of Veterinary Medicine farm and cow feces at the smallholder farms (Lushoto and Rungwe), respectively were assembled together and one Krona graph was made for each group. The enteric bacterial pathogens were identified through a literature search using dendrograms at the scholarly Google database.

Identification of antimicrobial resistance genes in the enteric bacterial pathogens

The characterization and annotation of resistant genes responsible for antimicrobial resistance in the bacterial pathogens were carried out using the Abricate database. The Abricate annotation pool used the ResFinder-2.1 dataset to identify and annotate potential antimicrobial resistance genes using BLAST similarity search (https://github.com/tseemann/abricate). The Abricate using the ResFinder selects the percentage identity (ID) thresholds that are identical between the best matching resistance genes and the corresponding sequence in the genome (Zankari et al., 2012). The default ID is 100%. These provided the type of antimicrobials in which are present and the accession numbers in the GenBank. Additionally, the pathogenic bacterium with multidrug resistance was identified by accessing the GenBank number provided by the ResFinder-2.1 in the National Center of Biotechnology Information (NCBI).

RESULTS

Characterization of enteric bacterial pathogens of economic importance identified in the rumen fluid and feces of cows raised at the station and smallholder farms

The study identified a high prevalence of enteric bacterial pathogens. These pathogens were characterized using dendrogram representations of rumen fluid and fecal samples. The bacterial species in each bacterial family present in the dendrograms were reviewed using scholarly Google database to identify those existing in rumen fluid and feces. Within each family, the highest number of bacterial species was detected in *Enterobacteriaceae* (five species) followed by *Streptococcaceae* and *Campylobacteriaceae* (three species each), *Staphylococcaceae* and *Enterococcaceae* (two bacterial species each) (Table 1). Both *Streptococcaceae* and *Enterococcaceae* families had two bacterial species detected in the rumen fluid and all the fecal samples. The *Enterobacteriaceae*, *Bacteroidaceae*, *Bacillaceae*, and *Prevotellaceae* had one species each detected in all the samples. The *Staphylococcaceae*, *Clostridiaceae*, and *Listeriaceae* were isolated from all the samples at the smallholder farms, whereas the *Mycoplasmataceae* and *Campylobacteriaceae* (*Campylobacter coli*) were only identified at the UoN Faculty of Veterinary Medicine farm samples. The bacterial species present in the fecal samples at the station farm were also detected in the rumen fluid, except *Campylobacter fetus, Shigella flexneri, Mycoplasma pneumoniae* and *Vibrio cholera.* More bacterial species were detected in fecal samples from smallholder dairy cows in Rungwe than in Lushoto. Specifically, *Streptococcus pneumoniae*, *Shigella dysenteriae* and *Clostridium perfringens* were only detected in fecal samples from Rungwe farm (Figure 1).

The enteric bacterial pathogens of economic importance were also determined by checking the number of counts (hits in the contigs) of bacterial pathogens through opening the dendrogram representations at the bacterial species level. The bacterial pathogens from bacterial families of *Enterobacteriaceae*, *Campylobacteriaceae*, *Bacteroidaceae*, and *Prevotellaceae* indicated the highest number of contigs in their genomes in all the samples. Bacterial pathogens from the feces recorded a high number of contigs than the rumen fluid samples. This is in agreement with the number of bacterial pathogens detected also both in feces and rumen fluid. Pathogenic species *Escherichia coli* and *Prevotella ruminicola* displayed the highest number of counts (185 and 220 respectively) in feces at Rungwe smallholder farms and rumen fluid samples respectively. Within the feces, samples from Tanzania had a relatively higher abundance in bacterial pathogens compared to those from the UoN station farm. At the Tanzania smallholder farm level, the bacterial pathogens detected in fecal samples from Rungwe indicated a relatively higher abundance than those sampled from Lushoto (Table 1).

Antimicrobial resistance (AMR) genes identified in the enteric bacterial pathogens

All the contigs (assembled genomes) were blasted against the ResFinder-2.1 database at the center of Abricate and the AMR genes were identified based on the similarity of genes present in the GenBank database. The results obtained show that there were 97 resistance genes in cow fecal samples and 8 resistance genes in the rumen fluid at UoN Faculty of Veterinary Medicine farm. The analysis of the fecal samples from the smallholder farms in Tanzania revealed a total of 295 and 307 resistance genes in Lushoto and Rungwe sites, respectively. The most abundant resistance genes detected in the enteric bacterial pathogens in this study confer resistance to beta-lactam (21 genes) and tetracycline (17 genes) drugs. The rest are shown in Table 2. The tetracycline and beta-lactam resistance genes were detected in all the fecal samples.

Table 1. Number of contigs with enteric bacterial pathogens of economic importance identified in the rumen fluid and feces of cows kept at the station (University of Nairobi) and smallholder (Lushoto and Rungwe) farms.

Bacterial family	Bacterial species	Rumen fluid*	Feces		
		UoN**	UoN**	Lushoto**	Rungwe**
Enterobacteriaceae	*Escherichia coli*	2	3	40	185
	Salmonella enterica	-	-	5	9
	Klebsiella pneumonia	-	-	2	19
	Shigella flexneri	-	1	1	16
	Shigella dysenteriae	-	-	-	5
Streptococcaceae	*Streptococcus agalactiae*	1	3	1	1
	Streptococcus pyogenes	1	2	2	1
	Streptococcus pneumonia	-	-	-	1
Campylobacteriaceae	*Campylobacter fetus*	-	6	41	27
	Campylobacter coli	1	8	-	-
	Campylobacter jejuni	-	-	37	31
Staphylococcaceae	*Staphylococcus aureus*	-	-	3	3
	Staphylococcus sciuri	-	-	1	2
Enterococcaceae	*Enterococcus faecium*	1	3	12	2
	Enterococcus faecalis	1	5	7	5
Clostridiaceae	*Clostridium botulinum*	-	-	21	4
	Clostridium perfringens	-	-	-	3
Bacteroidaceae	*Bacteroides fragilis*	39	45	16	50
	Bacteroides pyogenes	-	-	6	-
Bacillaceae	*Bacillus cereus*	2	6	1	5
Prevotellaceae	*Prevotella ruminicola*	220	18	5	10
Mycoplasmataceae	*Mycoplasma pneumonia*	-	1	-	-
Listeriaceae	*Listeria monocytogenes*	-	-	2	1
Vibrionaceae	*Vibrio cholera*	-	1	1	1

*Specimens of rumen fluid only collected from the University of Nairobi (UoN) station farm; **Number of contigs of bacterial specie in the sample;
**-: absence of bacterial specie in the sample.

Streptomycin, Sulfamethoxazole, Quinolone and Chloramphenicol resistance genes were only detected and highly prevalent in the smallholder farms.

Characterization of the drug resistant bacterial pathogens identified in the GenBank

The drug resistant bacterial pathogens were identified and characterized according to the similarity search in the GenBank. The antimicrobial resistant bacterial pathogens isolated from the cow feces at the UoN Faculty of Veterinary Medicine farm and smallholder farms in Tanzania are reported in Table 3. The most prevalent drug resistant bacterial pathogens were detected from *Enterobacteriaceae*, *Streptococcaceae*, *Campylobacteriaceae*, *Staphylococcaceae* and *Enterococcaceae*.

DISCUSSION

The highest number of bacterial species was detected in the *Enterobacteriaceae* followed by *Streptococcaceae*, *Campylobacteriaceae*, *Staphylococcaceae* and *Enterococcaceae* and the rest of the families had a species each (Table 1). The predominant *Enterobacteriaceae* and *Streptococcaceae* bacterial families are Gram negative and Gram positive respectively and are associated with brucellosis, pneumonia, salmonellosis, clinical and subclinical mastitis diseases predominantly reported in smallholder farms in the tropics. There are economically important diseases of livestock causing reproductive wastage through infertility, delayed heat, loss of calves, reduced meat and milk production, culling and economic losses from international trade bans of infected dairy products (Hossain et al., 2017). The *Staphylococcaceae*,

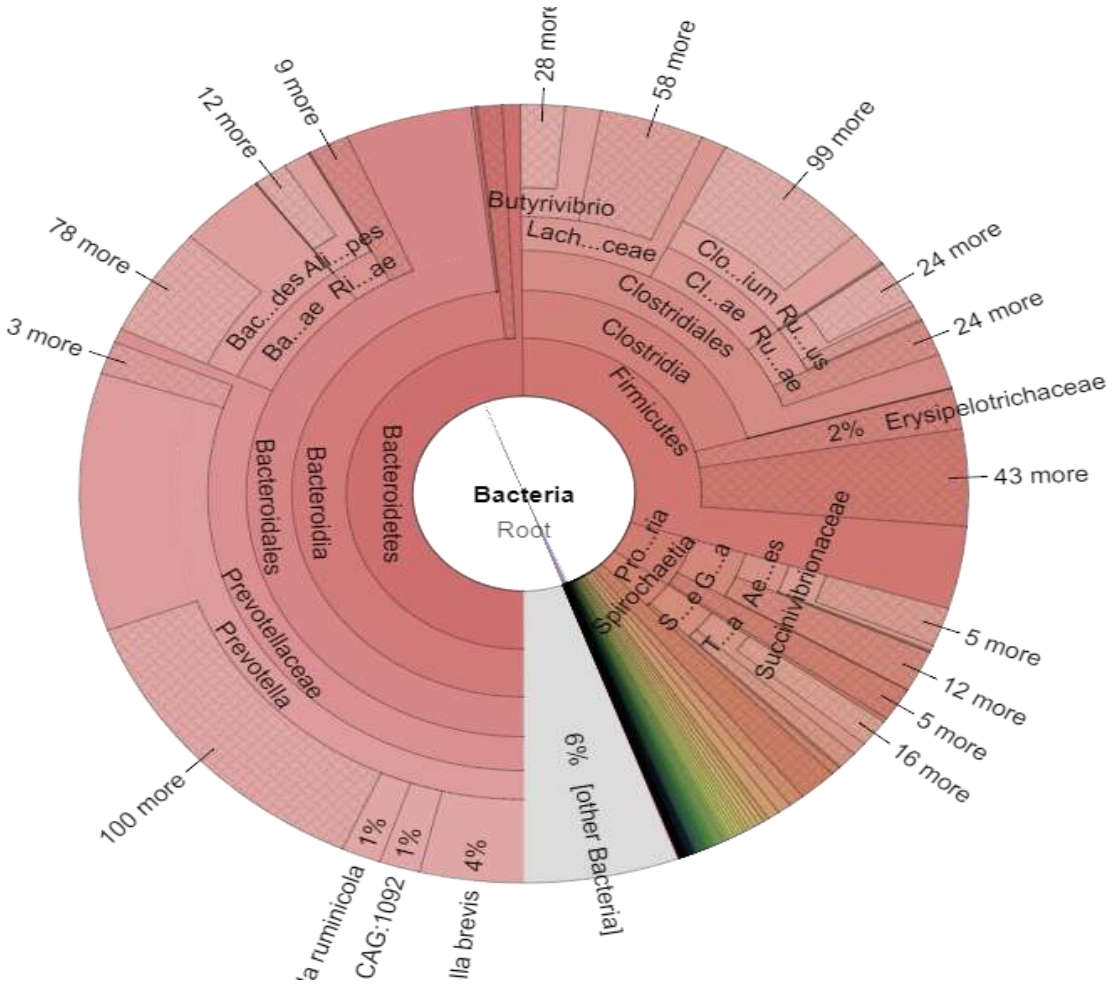

Figure 1. The taxonomic characterization of the rumen fluid bacterial species identified at the UoN Faculty of Veterinary Medicine farm.

Clostridiaceae and *Listeriaceae* bacterial families were detected in samples from smallholder farms, whereas the *Mycoplasmataceae* (*Mycoplasma pneumoniae*) and *Campylobacteriaceae* (*Campylobacter coli*) were identified at the station samples. There were differences observed between rumen fluid and feces, and also within fecal samples. The major bacterial pathogens identified from smallholder farms in this study, which included *Salmonella enterica*, *Klebsiella pneumoniae, Streptococcus sciuri*, *Campylobacter jejuni,* and *Staphylococcus aureus*, among others were in agreement with the findings reported by Osman et al. (2009) and Sharif and Muhammad (2009).

Additionally, these pathogens have been reported to cause clinical and subclinical mastitis (Idriss et al., 2014; Thompson-crispi et al., 2014; Abebe et al., 2016). The high presence of the enteric bacterial pathogens in the feces than the rumen fluid collected from the same cow indicates that most bacterial pathogens colonize the lower sections of gastro-intestinal tract of the animal such as colon, cecum, ileum and jejunum (Gerzova et al., 2015).

In the *Enterobacteriaceae*, *Escherichia coli*, *Salmonella enterica* and *Klebsiella pneumonia* was detected*. E. coli* is among the zoonotic bacterial pathogens that cause subclinical mastitis and commonly affects dairy cows during parturition leading them to the local or acute mastitis (Sandra Bjork, 2013; Osman et al., 2014; Madoshi et al., 2016; Hintong et al., 2017). It is excreted in the feces of healthy animal and spreads to the farm via soil or water (Amadi et al., 2015). This pathogenic bacterium was also identified in the cow fecal samples in other studies by Pandey at al. (2015), Madoshi et al. (2016) and Bako et al. (2017). Furthermore, *E. coli* were identified also by Megersha et al. (2009) and Amadi et al. (2015) in Ethiopia and Grenada in the feces of sheep and goats, respectively. In the present study, *K. pneumoniae* was detected as well. This bacterial pathogen was detected also in fecal samples of dairy cattle in other studies by Munoz et al. (2007), Sandra Bjork (2013),

Table 2. Number of contigs with antimicrobial resistance genes identified in the feces of cows kept at the station (University of Nairobi) and smallholder (Lushoto and Rungwe) farms.

Antimicrobial drug	Gene	Number of contigs (counts)		
		UoN	Lushoto	Rungwe
Tetracycline	*tet(32), tet(34), tet(35), tet(36), tet(37), tet(40), tet(44), tetA(P), tet(A), tet(C), tet(G), tet(H), tet(O), tet(Q), tet(S), tet(X), tet(W)*	56	64	89
Beta-Lactam	*blaACC-1, blaACC-2, blaBES-1, blaCMY-19, blaCMY-110, blaFAR-1, blaGOB-17, blaOXA-2, blaOXA-50, blaOXA-141, blaOXA-164, blaOXA-209, blaOXA-347, blaPAO, blaRHN-1, blaTEM-102, blaTEM-111, cfxA, cfxA2, cfxA3, cfxA6*	24	18	25
Streptomycin	*StrA, StrB , ant(6)-Ib, aadA17, aadE, aadK*	-	80	73
Sulfamethoxazole	*Sul1, Sul2*	-	37	35
Quinolone	*QnrB4, qepA, oqxB*	-	35	33
Chloramphenicol	*CatA, CatB, cmlA, floR*	-	27	25
Streptomycin and spectinomycin	*aadA1, aadA2, aadA4, aadA6*	-	11	9
Vancomycin	*VanG, VanR-D, VanR-F, VanR-G, VanS-G, VanY-Pt, vat(B), vat(E)*	1	5	3
Trimethoprim	*dfrB1, dfrG*	-	4	4
Lincomycin	*Lnu(C)*	5	-	1
Gentamicin	*aac(6')-aph(2'')*	2	1	2
Neomycin	*aph(3')-Ia, aph(3')-Ic*	-	3	2
Erythromycin	*Ere(A), erm(F)*	-	2	2
Oxazolidinone and phenicols	*optrA*	-	1	1

*Rumen fluid: Eight (8) resistance genes detected from bacterial pathogens in the rumen fluid at the University of Nairobi (UoN) were not reported because of their low abundance; -: absence of resistance genes.

Mansour et al. (2014) and Osman et al. (2014).

In the *Streptococcaceae*, *Streptococcus agalactiae* and *Streptococcus pyogenes* were detected in all the samples while *S. pneumoniae* was only present at Rungwe on-farms (Table 1). The Streptococcal species identified in the present study were lower than those reported by Mekibib et al. (2010) when studying bacterial pathogens causing mastitis in dairy cattle farms in Central Ethiopia. In the *Campylobacteriaceae*, *C. coli, C. fetus*, and *C. jejuni* were detected in cow rumen fluid and feces (Table 1). Similar findings were reported in Tanzania, Kenya and Ghana by (Kashoma et al., 2015; Nguyen et al., 2016; Karikari et al., 2017) in beef cattle feces, faeces and cloacal swabs of chickens and faeces and carcasses of healthy livestock animals, respectively. Pathogenic bacteria *Staphylococcus aureus* and *Staphylococcus sciuri* were detected only at the smallholder farms in Tanzania (Table 1). These bacterial pathogens which cause clinical and subclinical mastitis were also detected in the fecal samples in other studies by Mekibib et al. (2010), Sandra Bjork (2013) and Abo-Shama (2014). Finally, *Enterococcus* and *Clostridial species* were also detected. The pathogenic *E. faecalis* and *E . faecium* were detected both in the rumen fluid and feces. Similar findings were also reported by Goskel et al. (2016) and Beukers et al. (2017). They were also reported infecal and cecal samples of chickens by Diarra et al. (2010). Furthermore, *Clostridium botulinum* and *Clostridium perfringens* were detected in fecal samples from the smallholder farms. These findings are in agreement with the results reported by Ahsani et al. (2010), Kruger et al. (2011), Neuhaus et al. (2015) and Fohler et al. (2016) in animal feces and liquid manure from dairy cows.

In the current study, the antimicrobial resistance genes identified and characterized from the station and on-farms were presented in Table 2. The abundant resistant genes identified, confer resistance to tetracycline, beta-lactam, streptomycin, sulfamethoxazole, Quinolone and chloramphenicol drugs. Similar findings were reported by Thames et al. (2012) who reported the *tetC, tetG, tetO, tetW,* and *tetX* as antimicrobial resistance genes corresponding to tetracycline, *ermB, ermF* for macrolide, and *sul1* and *sul2* for sulfonamide identified from dairy calves manures. These findings are in agreement with the results by Agga et al. (2015), Gerzova et al. (2015), Iweriebor et al. (2015), Madoshi et al. (2016) and Pitta et

Table 3. Drug resistant bacterial pathogens of economic importance (including zoonotic) identified in feces of cows kept at station (University of Nairobi) and smallholder (Lushoto and Rungwe) farms.

Bacterial family	Bacterial species	Feces			Accession number
		UoN	Lushoto	Rungwe	
Enterobacteriaceae	*Escherichia coli*	-	+	+	AY224815
	Salmonella enterica	-	+	+	AY963803
	Klebsiella pneumonia	-	+	+	AB194410
	Shigella flexneri	-	+	+	AF321551
Streptococcaceae	*Streptococcus agalactiae*	+	+	+	AY928180
	Streptococcus pyogenes	+	+	+	AF227521
Campylobacteriaceae	*Campylobacter fetus*	+	+	+	FN594949
	Campylobacter jejuni	-	+	+	KF652095
Staphylococcaceae	*Staphylococcus aureus*	-	+	+	AJ579365
	Staphylococcus scriuri	-	-	+	U194559
Enterococcaceae	*Enterococcus faecalis*	+	+	+	AY271782
	Enterococcus faecium	+	-	+	KF421157
Clostridiaceae	*Clostridium perfringens*	-	-	+	L20800
Prevotellaceae	*Prevotella ruminicola*	+	+	+	L33696

+: Presence of drug resistant bacterial pathogens in the sample; -: Absence of drug resistant bacterial pathogens in the sample.

al. (2016) but are in contrast with the findings by Ahmed and Shimamoto (2011) and Chandra et al. (2014) who reported *blaCTX-M, blaTEM*, *blaCMY, blaSHV and blaOXA* as the predominant genes in their studies. The presence of a high number of genes that confer resistance to these antimicrobials can be explained by indiscriminate use of these drugs in the treatment of diseased animals, prevention of diseases in the farms or with their use as growth promoters in animal feed production (Sawant et al., 2007; Akindolire et al., 2015; Pandey et al., 2015; Cameron and McAllister, 2016). Furthermore, these drugs are cheap, widely available on the markets and have few predilection sites of administrations (Sawant et al., 2007; Osman et al., 2014; Beyene et al., 2017). The difference in the abundance of AMR genes was observed between the rumen fluid and cow feces isolates at the station farm (Table 2). The difference in AMR gene abundances also was observed between the isolates identified at the station and on-farms (Table 2). This difference could be due to differences in geographical locations, environment, management, farming practices, and concentration of the farms in the locations as reported by Kashoma et al. (2015) and Nyabundi et al. (2017). Additionally, the samples originating from different environment display different AMR gene abundance as reported by Gerzova et al. (2015). The present study identified the abundance of tetracycline resistance genes from cow fecal isolates. These findings are in agreement with the results by Thames et al., (2012), Agga et al. (2015), Gerzova et al. (2015), Iweriebor et al. (2015), Madoshi et al. (2016) and Pitta et al. (2016) who reported high presence of *tetC, tetG, tetO, tetW,* and *tetX* in their studies. The findings are also similar to the results reported by Kyselkova et al. (2015) when studying the occurrence of tetracycline resistance genes at conventional dairy farm. However, Englen et al. (2006) reported that *Campylobacter jejuni* displayed tetracycline and nalidixic acid resistance genes while *C. coli* indicated resistance to azithromycin, ciprofloxacin, clindamycin, erythromycin, gentamicin, and tetracycline from cow fecal isolates.

Moreover, beta-lactam resistance genes were also identified in the present study. The commonly identified AMR genes from cow fecal microbiota at the UoN Faculty of Veterinary Medicine farm were beta-lactam resistance genes (*cfxA_1, cfxA2_1, cfxA3_1 and cfxA6_1).* The high presence of *cfxA2*, and *cfxA3* resistance genes are in agreement with the findings by Chambers et al. (2015). The smallholder farms were dominated by bacteria showing beta-lactam resistance genes other than that detected at the station farm (Table 2). These findings are in agreement with the results reported by Ahmed and Shimamoto (2011), Jiang and Zhang (2013), Chandra et al. (2014) and Olowe et al. (2015). Furthermore, these findings are in contrast with the results reported by Mir et al. (2016) who reported high abundance of *blaTEM* and *blaCTX-M* genes detected from cefotaxime resistant bacteria in the cow feces. In this study, there was a difference between resistance genes identified at Lushoto and Rungwe smallholder farms. But no difference was

observed between Rungwe and UoN Faculty of Veterinary Medicine farms (Table 2). In the study on the prevalence of *E. coli* from dairy cattle feces in Eastern Cape, Iweriebor et al. (2015) reported that *E. coli* displayed resistance genes (*blaampc, blaCMY, blaCTX-M, blaTEM, tetA,* and *strA*) conferring resistance to beta-lactam, tetracycline, and streptomycin drugs, respectively. Similar findings were also reported in the current study. In the studies by Wittun et al. (2010), Schmid et al. (2013) and Gao et al. (2015), *E. coli* exhibited high presence of *CTX-M-14, CTX-M-15* and *TEM-52* genes from feces of dairy cattle and pig farms, respectively.

The findings from the present study revealed that streptomycin resistance genes (*StrA, StrB, ant(6)-Ib, aadA17, aadE, aadK)* were only present in the smallholder farms. These findings are in agreement with those reported by Srinivasan et al. (2007) in soils contaminated with bacterial pathogens in the dairy farm. The presence of *StrA* and *StrB* resistance genes in the current study are in agreement with the results reported by Aslam et al. (2010) from the cow feces infected with *E. coli* in Alberta, Canada. However, two sulfamethoxazole resistance genes which are *sul1* and *sul2* genes were also detected at the smallholder farms (Table 2). These findings are in agreement with the results by Gerzova et al. (2015) who reported the abundance of *strA, sul1*, and *sul2* genes in porcine fecal microbiota. In the present study, *QnrB4, qepA, oqxB* quinolone resistance genes were detected at the smallholder farms in Tanzania (Table 2). These findings are in contrast with the results reported by Zhang et al. (2015) who reported *qnrA* and *qnrS* genes detected in the pig fecal microbiota. However, no quinolone resistance gene was detected from cow fecal isolates at the UoN Faculty of Veterinary Medicine farm. No difference in abundance of quinolone resistance genes was observed between the isolates detected from cow feces at smallholder farms in Tanzania. In the study by Bae et al. (2005), the *C. jejuni* exhibited resistance to doxycline and *C. coli* showed resistance to quinolone antimicrobials detected also in the current study.

In conclusion, *CatA, CatB, cmlA, floR* chloramphenicol resistance genes from cow fecal isolates were identified in the smallholder farms (Table 2). Similar findings were reported by Sudda et al. (2016) in Tanzania. However, there was no chloramphenicol resistance gene identified at the station farm. The high abundance of chloramphenicol resistance genes at smallholder farms could be due to the smallholder farmers using the non-prescription drugs and not keeping treatment records about animals. Additionally, this presence could be attributed to its widespread and indiscriminate use in the treatment and prevention of diseases, or transfer of the resistant genes between animals, humans, and environments through the cross contamination (Omojowo and Omojasola, 2013; Sudda et al., 2016; Beyene et al., 2017; Messele et al., 2017).

Conclusion

This study results deepens our understanding of the diversity of enteric bacterial pathogens detected from cow rumen fluid and feces at the UoN Faculty of Veterinary Medicine farm and smallholder farms. It provided also insight on the prevalence of AMR genes from those enteric bacterial pathogens in the dairy cows whose cow feces is used to fertilize the farms. The characterized enteric bacterial pathogens of economic importance include *E. coli, S. enterica, K. pneumoniae, S. agalactiae, S. pyogenes, C. coli, C. fetus, C. jejuni, S. aureus, S. sciuri, E. faecalis, E. faecium, C. botulinum* and *C. perfringens*. Furthermore, the resistant genes detected in the enteric bacterial pathogens in this study confer resistance to tetracycline (17 genes from 209 contigs), beta-lactam (21 genes from 67 contigs), streptomycin (6 genes from 153 contigs), sulfamethoxazole (2 genes from 72 contigs), Quinolone (3 genes from 68 contigs) and chloramphenicol (4 genes from 52 contigs). Therefore, the identification of genes responsible for antimicrobial resistance in the bacterial pathogens may allow the development of novel clinical interventions against the GIT diseases of the dairy cows. The future studies are needed to identify the drug resistant bacterial pathogens with the spread of antimicrobial resistance in the farms. This will become a clear tool for developing the strategy to prevent the indiscriminate use of already resistant drugs in the farms.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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