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Unravelling Antimicrobial Resistance Phenotypes and Carriage of Extended-Spectrum β-Lactamase Genes in *Escherichia coli* Isolated from Dairy Farms in Kiambu County, Kenya

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Abstract

The use of antibiotics for prophylaxis and growth enhancement in livestock farming is on the increase globally. This practice has led to the emergence and spread of antimicrobial-resistant bacteria in livestock. Only limited research has been done to establish the role of cattle farming in antimicrobial resistance. The current study sought to establish the carriage of multi-drug resistance and extended-spectrum beta-lactamase genes in *Escherichia coli* from farmers, their cattle, and cattle slurry within Kiambu County. A total of 286 (81%) *E. coli* isolates were recovered from 352 samples analysed. Antibiotic resistance profiles showed 114 (40%) isolates were resistant to ≥3 antimicrobial classes and were considered multidrug-resistant. Among multidrug-resistant (MDR) *E. coli* strains, 40 (14%) were resistant to 3 different antimicrobial classes, while 71 (25%) were resistant to between 4 and 7 antibiotic classes. Extended-spectrum β-lactamase resistance was found in 18 isolates: human (n = 14), cattle (n = 2), and environmental (n = 2). Both the bla_{CTX-M} and bla_{TEM} genes were detected in 10 and 15 strains, respectively. Sequence analysis showed that the isolates carried the $bla_{TEM-116}$ (n = 7), bla_{TEM-1} (n = 5), and $bla_{CTX-M-15}$ (n = 8) genes. Genotyping MDR isolates using $(GTG)_5$ PCR demonstrated that the isolates were not clonal. This data shows antimicrobial resistance profiles and different types of resistance genes in the *E. coli* population on dairy farms. As a result, more effective, targeted public health policies and measures need to be put in place to control and prevent the emergence and spread of resistant bacteria.

Keywords

Humans, Cattle Slurry, *Escherichia coli*, Multidrug Resistance, Extended-Spectrum β-Lactamase (ESBL), TEM 116, CTX-M-15, Kenya

1. Introduction

Veterinary antibiotics are progressively associated with major health issues in the animal health and welfare sector globally. These health issues could be attributed to the increasing antibiotic resistance build-up noted in animal pathogens [1] [2]. In Kenya, veterinary practice and livestock production have a mean antibiotic consumption of 14,594 ± 1457 kilograms per year [3]. About 60% (between 30% and 90%) of the antibiotics used in veterinary medicine are not entirely metabolised when eliminated through urine or faecal matter [4]. The antibiotic residue from manure could impact the structure and function of microbial communities and promote the spread of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) [5].

Escherichia coli (*E. coli*) is among the seven species highlighted as key antimicrobial-resistant bacteria used as a sentinel pathogen for antimicrobial resistance development in human beings and animals [6]. It is possible that *E. coli*, which is a common microbiota in the digestive tract, can pass on resistance to other bacterial species. This is because *E. coli* can be pathogenic, and it has been known to develop antimicrobial resistance [7] [8] [9].

The rise and spread of extended-spectrum β-lactamase-producing *E. coli* linked to cattle and other livestock have been a particular source of concern [10] [11]. Although clavulanic acid inhibits extended-spectrum β-lactamases (ESBLs), the ability of these enzymes to hydrolyse third-generation cephalosporins and monobactams is common. The spread of different types of ESBLs has changed so far, with a sudden rise in CTX-M enzymes, which are found in the human population, over SHV and TEM variants [12]. The accelerated development and spread of ESBLs in pathogenic *E. coli* isolate in agricultural farms pose a major problem in treating infections in healthcare facilities because the pathogens are often resistant to aminoglycosides, trimethoprim-sulfamethoxazole, and fluoroquinolones.

In Kiambu, like many rural areas in Kenya, animal manure is extensively used in crop production as a source of variable plant nutrients. Therefore, farmers are constantly exposed to bacterial strains contained in manure and easily ingest them, especially if proper hygiene is not adhered to. In Africa, data on linkages between bacterial strains recoverable from animal droppings/manure and strains in the human population remains scarce. There is high-intensity mixed farming in these sites, with decreasing farm sizes due to increasing population growth. The commonly used agricultural soil amendment in Kiambu County is cattle slurry, which exhibits a possible transmission pathway of antimicrobial-resistant bacteria (ARB) to the human population. Some of the most critical methods of

transmission could be through hands, water, and food contamination.

This study sought to determine the carriage of multidrug-resistant *E. coli* and the distribution of ESBL resistance in *E. coli* in the farming community at the human-livestock-environmental interface. The data provided in-depth knowledge of how farms and animal excreta contribute to the persistence of antimicrobial resistance in both the environment and the human population. The fundamental information provided would help understand the role of farmers, livestock, and farm waste as a reservoir of resistance on agricultural farms.

2. Materials and Methods

2.1. Study Population and Design

In this study, 88 randomly selected small-scale dairy farms were enrolled from four sub-counties of Kiambu, including Lari, Githunguri, Gatundu North, and Kiambaa sub-counties, between January and March 2020. The number of dairy farms sampled from each sub-county was determined by proportional allocation. From each farm, non-human samples comprising a fresh faecal sample from each farm were collected from the participating zero-grazed cattle. A slurry sample was also obtained from the farm's slurry tank. The study obtained human samples with informed consent from farmers who provided fresh faecal materials and hand swab samples. Each sub-county had an equal number of households selected. One dairy farm was randomly selected, and one sample of each type was aseptically collected from every sub-location. Dairy farms in each sub-county had to meet strict inclusion criteria, including having cattle at the time of sampling, and the farmer who provided the samples was working intensively in the dairy unit or the field spread with manure.

2.2. Sample Processing

All fresh faecal and slurry samples were put into Cary-Blair media and transported on ice to the KEMRI-CMR laboratory. Hand swabs from the farmworkers were placed in Amies transport media. In all cases, samples were kept at 4˚C during transit, and isolation of *E. coli* started within 6 h of collection. Isolation from faecal and slurry samples involved direct culture on MacConkey's agar (Oxoid, UK) and Eosin Methylene Blue Agar (EMBA) (Oxoid, UK). For hand swab samples, buffered peptone water was used for 18 h to enrich before sub-culturing onto MacConkey's agar and EMBA.

A single colony of rapid fermentation (pink-burgundy colour) was randomly picked from the plate for each original sample and tested for indole production and oxidase activity. Indole-positive, oxidase-negative strains were presumptively considered *E. coli*. All selected isolates were run on API 20e multi-test strips (Biomerieux, USA). All confirmed *E. coli* isolates were immediately stored at −80˚C in Tryptic-soya broth agar (with 15% glycerol) and were grown on Mueller Hinton Agar (MHA) from frozen stocks for each subsequent characterisation.

2.3. Antibiotic Susceptibility Test

Disc diffusion antibiotic sensitivity tests were carried out following the Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. Briefly, bacterial colonies were picked from MHA plates, initially inoculated, and incubated for 24 h at 37˚C. Each colony sample was emulsified in normal saline until it reached 0.5 McFarland standard concentration before being seeded onto Mueller Hinton (MH) plates. Then, the antibiotics were placed onto the plate surface, with five antibiotic discs per plate (Table 1). The measured inhibition zones were interpreted as per the standard measurement table [13]. For consistency, antibiotic

Table 1. Antibiotic assay discs, abbreviations, and amount of antibiotic in each disc.

Antibiotics	Content
Penicillin	
Ampicillin (AMP)	10 µg
Amoxicillin-clavulanic acid (AMC)	20/10µg
Cephalosporin	
Cefotaxime (CTX)	30 µg
Ceftazidime (CAZ)	30 µg
Ceftriaxone (CRO)	30 µg
Cefepime (FEP)	30 µg
Cephamycin	
Cefoxitin (FOX)	30 µg
Carbapenem	
Meropenem (MEM)	10 µg
Aminoglycoside	
Streptomycin (S10)	10 µg
Amikacin (AK)	30 µg
Kanamycin (K)	
Quinolones	
Ciprofloxacin (CIP)	5 µg
Sulphonamide/complex	
Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75µg
Sulfamethoxazole (RL)	25 µg
Trimethoprim (W)	5 µg
Phenicol	
Chloramphenicol (C)	30 µg
Tetracycline	
Tetracycline (T)	30 µg

resistance scores of less than 6 mm (nominal disc size in millimeters) were replaced with 6 mm as a minimum score. Following previous studies, intermediate strains were deemed to be moving towards resistance and thus considered resistant on an evolutionary basis [14]. Therefore, a strain found to be non-susceptible to at least three or more antimicrobial classes was scored as an MDR strain [15]. *E. coli* ATCC 25922 (ESBL negative) and *E. coli* NCTC 13,353 (ESBL positive CTXM-15) were used as quality control strains.

2.4. Phenotypic Confirmation of ESBL Producing *E. coli*

All isolates that gave an inhibition zone indicating resistance or intermediate resistance to Ceftazidime (CAZ) or Cefotaxime (CTX) using standard antibiotic discs were further tested for ESBL production using the BD Total ESBL Confirm Kit (BD, USA) following the guidelines of the manufacturer. Briefly, the combination of CTX and CAZ discs (30 µg of each antibiotic) alone and in a combination with clavulanic acid (CA) was performed. An increase in the diameter of the zone of clearing around a disc ≥ 5 mm for either antimicrobial agent tested in combination with CA versus the diameter of the zone of clearing around a disc containing the agent when tested alone indicated ESBL presence [13]. Control strains used were *E. coli* ATCC 25922 (ESBL negative) and *E. coli* NCTC 13353 (ESBL positive).

2.5. Genotyping of Isolates

2.5.1. Detection of *bla* (ESBL) Genes

Phenotypically confirmed ESBL-producing *E. coli* strains were examined further to detect the presence of bla_{SHV}, bla_{TEM}, bla_{OXA}, and $bla_{CTX\text{-}M}$ genes by Polymerase Chain Reaction (PCR) [16]. In brief, the test strains were cultured on Mueller Hinton agar for 18 hours at 37˚C to extract DNA using commercial kit (QIAamp DNA kit, Qiagen, Hilden, Germany) following manufacture's instructions. The purity and concentration of DNA were tested using a NanoDrop ND-1000 UV-vis spectrophotometer (NanoDrop Technologies, Wilmington, USA). Total DNA (2 µl) was used in a 20 µl reaction mixture that contained 4 µl of the Ready to Mix 5× FIREPOL® Master Mix (Solis Biodyne, UK), 12.2 µl of nuclease-free water, optimised 1 µl of BSA, and 0.4 µl of each primer. The primers and expected product sizes are given in **Table 2**. *E. coli* NCTC 13353 was used as a positive control for TEM, *Klebsiella pneumoniae* NCTC 13368 was used as a positive control for SHV, and *E. coli* ATCC 25922 was used as a negative control for PCR.

The cycling conditions included one cycle denaturation for 2 min at 95˚C, 35 cycles of 3 min at 95˚C, 1 min at 55˚C, and 5 min at 72˚C. The PCR reaction ended with a final extension step of 72˚C for 15 min.

Approximately 5 µl of PCR products were loaded onto horizontal 1.5% w/v agarose gel with a 1 kb plus molecular size marker (Invitrogen, UK) and electrophoresed at 80 V for 40 minutes. The gels were stained with SYBR green dye. The DNA bands were then visualised with a UV transilluminator (UVP Inc.), and the images were taken using black and white Polaroid film.

Table 2. List of PCR primers used for detection of β-lactamase genes (SHV, OXA, TEM, and CTX-M). Primer sequences and expected PCR product sizes are shown for each primer.

Oligonucleotide name	Primer sequence	Annealing temperature (˚C)	Product size (bp)
CTX-M-F-	ATGTGCAGYACCAGTAARGTKATGGC	56	529
CTX-M-R-	TGGGTRAARTARGTSACCAGAAYSAGCGG		
TEM-F-	GCGGAACCCCTATTT G	50	964
TEM-R-	ACCAATGCTTAATCAGTGAG		
SHV-F-	TTATCTCCCTGTTAGCCACC	50	796
SHV-R-	GATTTGCTGATTTCGCTCGG		
OXA-1-F-	ATGAAAAACACAATACATATCAACTTCGC	62	820
OXA-1-R-	GTGTGTTTAGAATGGTGATCGCATT		
OXA-2-F-	ACGATAGTTGTGGCAGACGAAC	60	601
OXA-2-R-	ATYCTGTTTGGCGTATCRATATTC		

R is a purine; Y is a pyrimidine; S is G or C.

2.5.2. Purification of PCR Products and Sequencing

PCR products of the isolates positive for *bla* genes were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and subjected to direct sequencing at Macrogen Europe (Amsterdam, Netherlands).

2.5.3. $(GTG)_5$-PCR Banding Pattern Analysis

E. coli isolates for fingerprint analysis were selected based on resistance profiles, sample type, and sampling area. Representatives of MDR isolates that were resistant to six or more classes of antimicrobials, ESBLs, and fully susceptible isolates were selected for analysis. The GTG5-PCR was done using a single primer (5'-GTGGTGGTGGTGGTG-3') under the following modified amplification conditions; denaturation step at 95˚C for 7 min followed by 30 cycles (1 min at 94˚C + 1 min at 40˚C + 8 min at 60˚C) and final elongation for 15 min at 65˚C [17]. The resulting PCR products were separated in 1.5% agarose gel electrophoresis at 80 V for 120 min and visualized under UV light.

2.6. Data Management and Statistical Analysis

All statistical analyses were performed using STATA v 13 (Stata Corp LP, College Station, TX, USA). Clustering of antibiotic profiles was carried out using WHONET 2020. Descriptive statistics; frequency and percentages (%) were used to describe antimicrobial susceptibility patterns and the distribution of MDR strains across different sources. For categorical variables, Chi-square was used to test for significance where applicable.

Diversity analysis of the resistant strains from both humans and the imme-

diate environment was done to determine the possible genetic diversity among recovered *E. coli* isolates. Generated *E. coli* electrophoresis images were analysed using Gelcompar® 2 software version 6.6 Bionumeric software and dendrogram tree plotted using the unweighted pair group method (UPGMA) with arithmetic mean using Pearson correlation. A similarity matrix of ≥80% among isolates in various generated dendrogram clusters was considered a significant indication of genetic similarity.

On the other hand, obtained chromatogram sequencing files were inspected and corrected using the software application GENtle v. 1.9.4 http://gentle.magnusmanske.de [18]. Multiple sequence alignments of the nucleic acid were carried out using the ClustalW program. The sequences were identified by comparing them with those available in the National Centre for Biotechnology Information database (NCBI) using the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) [19].

3. Results

3.1. Antimicrobial Resistance in Isolates from Clinical and Environmental Samples

In this study, a total of 352 samples were collected from dairy farms in Kiambu County, including 88 samples from each of the four sample types (human faecal, hand swabs, slurry, and fresh cattle dung). From the 352 samples analysed, 286 (81%) *E. coli* isolates (non-human samples (n = 163) and human samples (n = 123) were recovered. Out of 286 confirmed *E. coli* isolates, only 56 (20%) were sensitive to all antibiotics, whereas 230 (80%) showed resistance to at least one antibiotic (**Table 3**) (**Figure 1**). Of the total isolates, 114 (40%) were multidrug-resistant (MDR)

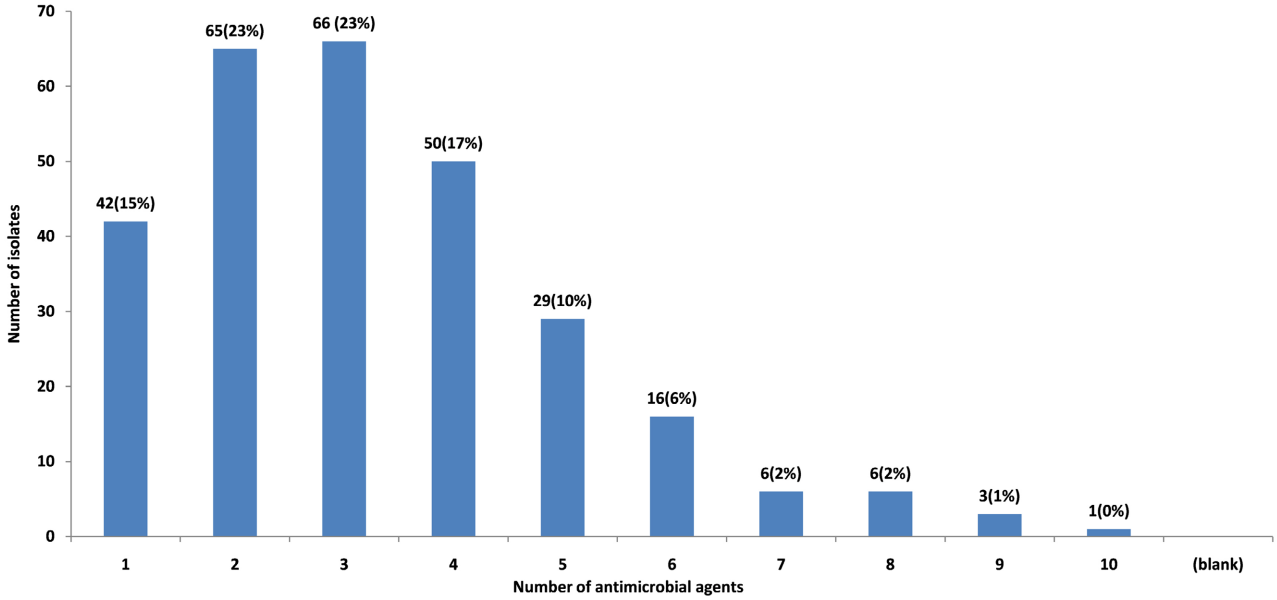

Figure 1. Overall frequency resistance to antimicrobial agents among *Escherichia coli* isolated from human faecal, hand swabs, slurry, and fresh cattle dung samples.

Table 3. Antimicrobial resistance profiles in *Escherichia coli* isolates from clinical, animal, and environmental samples.

Sample category	Sample type	Number of isolates	Antimicrobial resistance *Escherichia coli* n (%)																
			AMC	AMK	AMP	CAZ	CHL	CIP	CRO	CTX	FEP	FOX	KAN	MEM	SMX	STH	SXT	TCY	TMP
Livestock	Fresh cattle dung	83	3 (4)	18 (22)	21 (25)	5 (6)	5 (6)	3 (4)	4 (5)	4 (5)	4 (5)	6 (7)	0 (0)	0 (0)	54 (65)	24 (29)	7 (8)	26 (31)	13 (16)
Human	Hand swab	49	6 (12)	10 (20)	10 (20)	3 (6)	1 (2)	1 (2)	3 (6)	2 (4)	6 (12)	5 (10)	2 (4)	0 (0)	30 (61)	13 (27)	4 (8)	19 (39)	11 (22)
Human	Human faecal	74	8 (11)	10 (14)	19 (26)	5 (7)	2 (3)	1 (1)	6 (8)	3 (4)	8 (11)	3 (4)	3 (4)	0 (0)	52 (70)	22 (30)	5 (7)	24 (32)	15 (21)
Environmental	Slurry	80	1 (1)	11 (14)	23 (29)	4 (5)	3 (4)	1 (1)	4 (5)	2 (3)	4 (5)	8 (10)	1 (1)	1 (1)	52 (65)	26 (33)	9 (11)	24 (30)	19 (24)
	All	286	18 (6)	49 (17)	73 (26)	17 (6)	11 (4)	6 (2)	17 (6)	11 (4)	22 (8)	22 (8)	6 (2)	1 (0.3)	188 (66)	85 (30)	25 (9)	93 (33)	58 (21)

Key: Antimicrobial activity of *Escherichia coli* isolates was tested against 17 antibiotics against that includes; **AMP:** Ampicillin, **AMC:** Amoxicillin-clavulanic acid, **CRO:** Ceftriaxone, **FEP:** Cefepime, **FOX:** Cefoxitin, **MEM:** Meropenem, **AMK:** Amikacin, **KAN:** Kanamycin, **STH:** Streptomycin; **CIP:** Ciprofloxacin, **SXT**: Trimethoprim-sulfamethoxazole, **CHL:** Chloramphenicol and **TCY:** Tetracycline, **CAZ**: Ceftazidime; **CTX**: Cefotaxime, **TMP**: Trimethoprim, **SMX**: Sulfamethoxazole.

as earlier defined [15] and showed resistance to at least one antibiotic from three or more different classes (**Figure 2**). The highest number of the MDR isolates were recovered from human faecal samples, 39 (34%), followed by fresh cattle dung, 35 (31%), then slurry, 20 (18%), and hand swab samples, 20 (18%), respectively (**Figure 3**). The resistance profile of MDR strains ranged from 3 to 8 antimicrobial classes (**Figure 2**). The highest percentage of MDR isolates were resistant to antibiotics in three antimicrobial classes, 40 (14%), and 3 (1%) were resistant to agents in 8 classes. This study detected a significant difference in multiple antimicrobial resistance phenotypes in *E. coli* isolates from human and non-human samples (**P** = 0.000264, **O.R**: 6.375, **C.I**: 2.0871 - 19.472). In all isolates analysed, prevalent resistance was recorded towards sulfamethoxazole 188 (66%), tetracycline 93 (33%), and ampicillin 73 (26%). Meropenem and Kanamycin had the least frequent resistance, with only 1 (0.3%) and 6 (2%) resistances noted, respectively. Isolates analysed in this study were co-resistant to between 1 - 10 antimicrobial agents tested (**Figure 1**). However, a significant proportion of 66 (23%) of the isolates in this study were resistant to 2 or 3 antimicrobial agents (**Figure 1**). *E. coli* isolates from cattle slurry and human faecal samples had the most antimicrobial resistance combinations, with over 12% resistant to 2, 3, or 4 antimicrobial agents (**Figure 4**). The *E. coli* isolates from human faecal samples were resistant to most of the tested antimicrobial agents, ranging between 0% - 70%, followed by hand swab isolates with ranges of between 0% - 61% (**Table 3**). In non-human samples, isolates from the slurry sample were resistant to up to 7 antimicrobial agents ranging from1% - 65%.

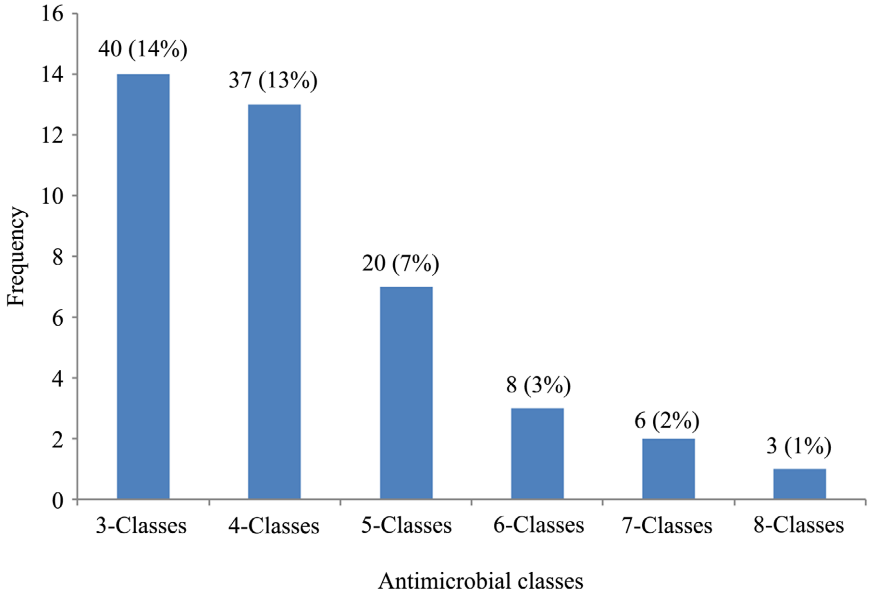

Figure 2. Number of antimicrobial classes co-resisted by MDR *Escherichia coli* isolated from human faecal, hand swabs, slurry, and fresh cattle dung samples.

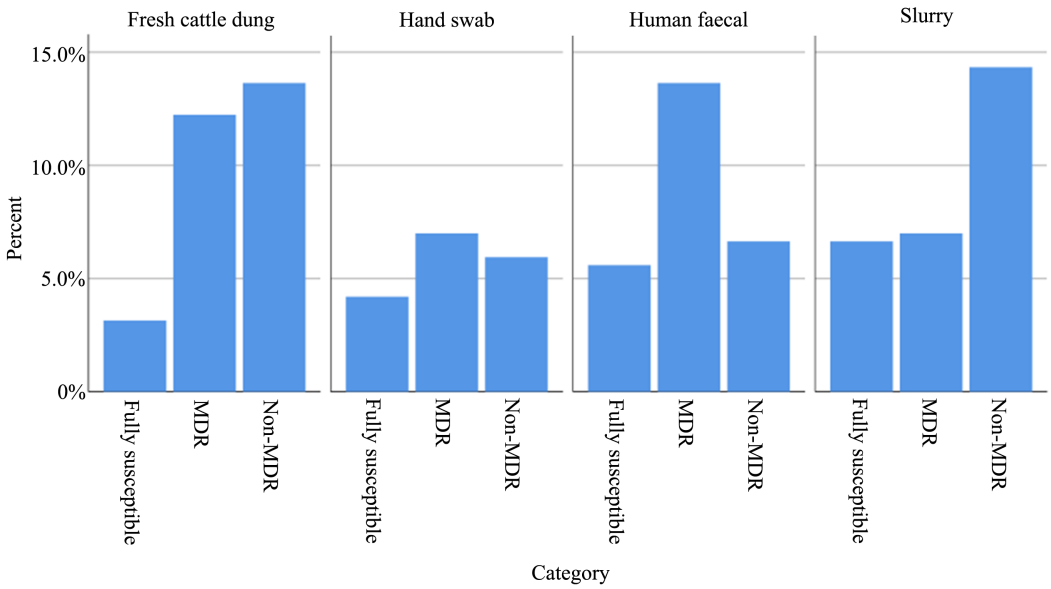

Figure 3. Percentage distribution of fully susceptible, non-MDR, and MDR *E. coli* isolates among human faecal, hand swabs, slurry, and fresh cattle dung samples.

E. coli isolates from fresh cattle dung were resistant to up to 6 antimicrobial agents with a range of 0% - 65% (Table 3).

3.2. Extended *β*-Lactamases Phenotype and Genotype

A total of 286 *E. coli* isolates were screened for antimicrobial activities, and 28 (10%) of these were resistant to either ceftazidime or cefotaxime (Table 3). These isolates were further analysed to confirm the ESBL phenotype. The confirmatory test kit showed that 15 (54%) of the 28 isolates were ESBL phenotypes

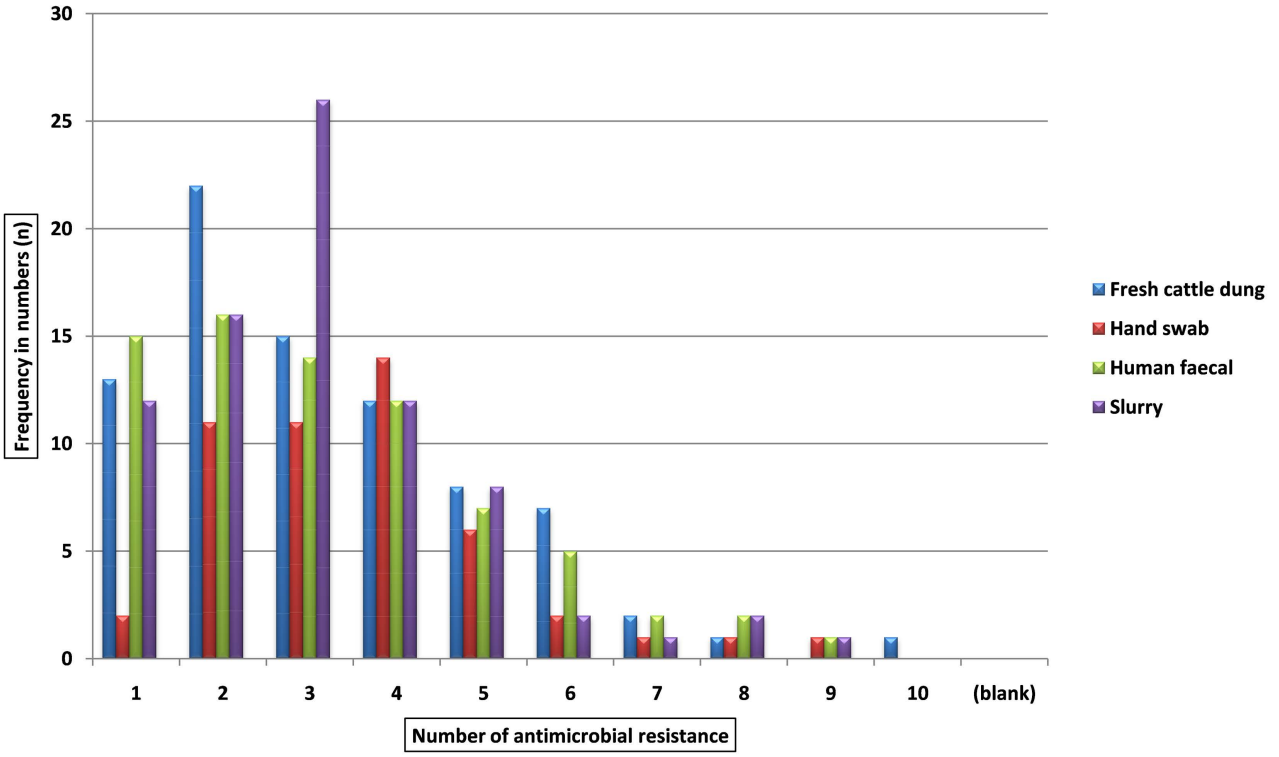

Figure 4. Frequency in numbers of antimicrobial agents resisted in *Escherichia coli* isolates from various samples types.

(**Table 4**). The ESBL phenotype was more common in human faecal isolates (7 (47%), followed by isolates in hand swabs (4 (26%), cattle dung (2 (13%), and Slurry 2 (13%).

The PCR test for *bla* genes carriage showed that 10 (56%) of the 18 screened ESBL producers were positive for $bla_{CTX\text{-}M}$. Fifteen (83%) isolates had bla_{TEM} genes (**Table 5**). The carriage of $bla_{CTX\text{-}M}$ and bla_{TEM} was common in isolates from human faecal samples, with 5 (50%) and 7 (47%), respectively (**Table 5**). In addition, 10 (67%) of the screened isolates were positive for carriage of both $bla_{CTX\text{-}M}$ and bla_{TEM}. None of the 18 confirmed ESBL producers was positive for carriage of OXA and SHV genes.

3.3. Heatmap Representation of Antimicrobial Resistance in Extended-Spectrum *β*-Lactam Phenotypes

In a heatmap generated, 4 (40%) of the *E. coli* isolates with TEM and CTX-M co-carriage clustered together, reflecting similarity in antimicrobial resistance profiles. Similar results were also noted where 6 (46%) of the fully susceptible isolates tested clustered together. Isolates that harboured the TEM gene only were, however, scattered in various clusters in the heatmap (**Figure 5**).

3.4. Sequences Analysis and Polymorphism

3.4.1. Analysis of CTX-M Gene ($bla_{CTX\text{-}M}$)

The CTX-M gene sequences from eight $bla_{CTX\text{-}M}$ positive isolates were 100%

Table 4. Double disk diffusion for confirmation of ESBL phenotype.

Isolate ID	Sample type	CAZ	CAZ + C	D	CTX	CTX + C	D	ESBL phenotype
5519_HDS	Hand swab	29	32	3	23	28	5	ESBL
5521_FCD	Cattle dung	23	24	1	27	27	0	Non-ESBL
5530_FCD	Cattle dung	27	29	2	23	26	3	Non-ESBL
5534_HDS	Hand swab	15	25	10	12	28	16	ESBL
5534_HFD	Human faecal	17	27	10	10	28	18	ESBL
5540_CDS	Slurry	25	28	3	25	27	2	Non-ESBL
5542_CDS	Slurry	20	31	11	32	32	0	ESBL
5548_FCD	Cattle dung	18	30	12	30	32	2	ESBL
5550_HFD	Human faecal	25	27	2	19	23	4	Non-ESBL
5551_FCD	Cattle dung	19	23	4	32	32	0	Non-ESBL
5552_HFD	Human faecal	18	22	4	30	30	0	Non-ESBL
5555_CDS	Slurry	19	31	12	30	31	0	ESBL
5555_HFD	Human faecal	17	28	11	11	28	17	ESBL
5556_HFD	Human faecal	18	27	9	7	29	22	ESBL
5558_HDS	Hand swab	20	30	10	13	32	19	ESBL
5558_HFD	Human faecal	0	12	12	9	9	0	ESBL
5559_HFD	Human faecal	20	30	8	13	30	17	ESBL
5560_HFD	Human faecal	20	26	6	12	27	15	ESBL
5561_HDS	Hand swab	30	32	2	18	32	14	ESBL
5562_HFD	Human faecal	27	29	2	20	25	5	ESBL
5569_HDS	Hand swab	24	27	3	24	28	4	Non-ESBL
5572_FCD	Cattle dung	26	27	1	25	28	3	Non-ESBL
5572_HDS	Hand swab	17	20	3	31	31	0	Non-ESBL
5576_HFD	Human faecal	17	28	11	0	28	28	ESBL
5577_HDS	Hand swab	18	28	10	8	30	22	ESBL
5577_FCD	Cattle dung	20	30	10	32	32	0	ESBL
5588_HDS	Hand swab	26	27	1	24	27	3	Non-ESBL
5590_HFD	Human faecal	17	22	5	31	31	0	ESBL
NCTC 13353	Control	12	27	15	0	27	27	ESBL
E. cloacae	Control	10	12	2	8	11	3	Non-ESBL

Phenotypic verification of the ESBL phenotype was done by measuring the differences in inhibition zone of a cephalosporin-inhibitor (CAZ + clav, CTX + clav) and cephalosporin (CAZ and CTX) antimicrobial disk. The description of various acronyms used in this table is as follows; **CAZ**: Ceftazidime, **CTX**: Cefotaxime, **clav**: clavulanic acid (bacterial growth inhibitor), **AMR**: Antimicrobial resistance, **ESBL**: Extended-spectrum β-lactamases, **FCD**: fresh cattle dung, **HDS**: Hand swab, **CDS**: Cattle dung slurry and **HFD**: Human faecal sample, **D**: Difference of ≥ 5 mm indicates ESBL presence.

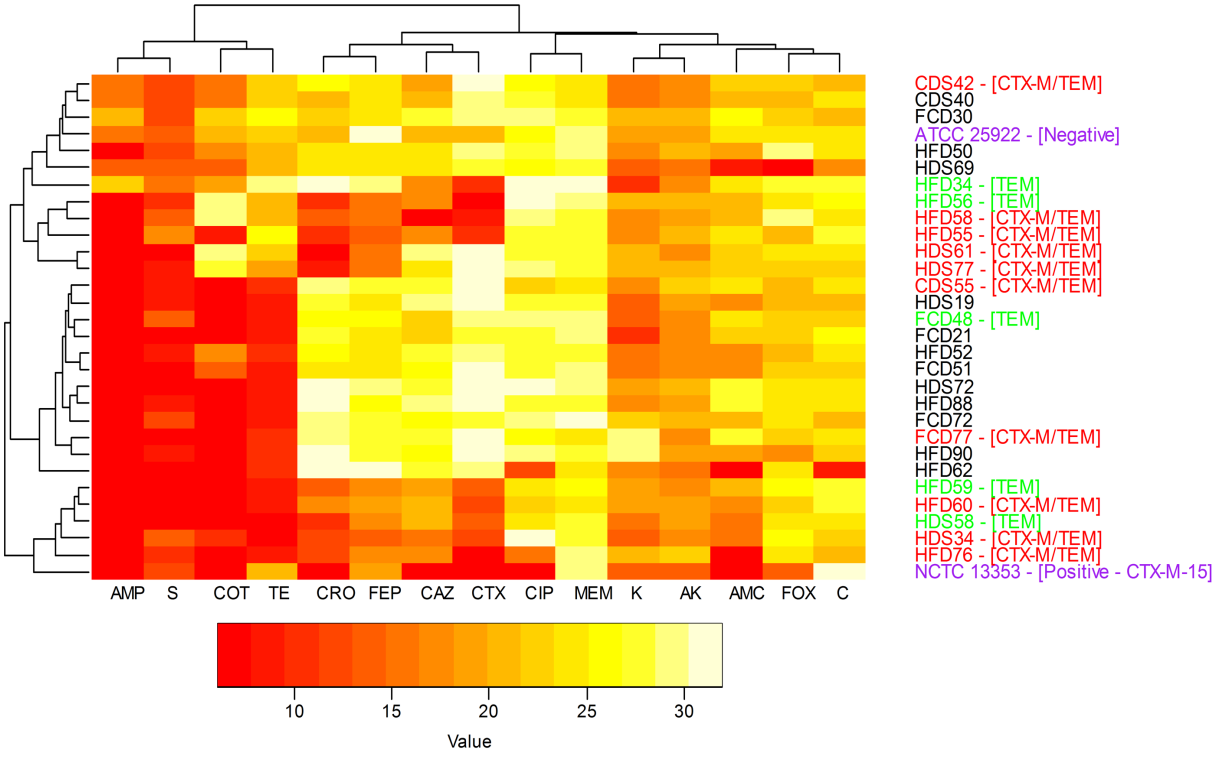

Figure 5. Heatmap representation of zone of inhibitions around antibiotic discs for 28 isolates that were phenotypically ESBL positive. The colour spectrum represents the size of inhibition zones in the diagram, with red representing high resistance patterns (no zone) while orange through yellow illustrates an increased zone of inhibition (resistant-zone of clearing 10 - 22 mm depending on antibiotic breakpoints). A zone of >30 mm is characterised by white colour and indicates high sensitivity. The presence of bla_{CTX-M} or bla_{TEM} is indicated by blue and green colour typefaces, respectively. Strains that have both genes (bla_{CTX-M} and bla_{TEM}) are denoted by red typeface. The heatmap was generated using Maximum distance and ward. D algorithmic methods.

Table 5. *β*-lactamases genes detected by PCR in *Escherichia coli* isolates.

Sample type	Number of isolates	ESBL-phenotype	*β*-lactamases genes in *E. coli* isolates			
			blaTEM	blaCTX-M	bla SHV	blaOXA
Human faecal	74	9 (12%)	7	5	0	0
Hand swabs	49	5 (10%)	4	3	0	0
Slurry	80	2 (3%)	2	2	0	0
Fresh cattle dung	83	2 (2%)	2	0	0	0
Total	286	18 (6%)	15 (83%)	10 (56%)	0	0

Polymerase chain reaction (PCR) was used to screen for the carriage of the common *β*-lactamase gene (bla) that includes the Temoneria (bla_{TEM}), Cefotaxime Munich (bla_{CTX-M}), Oxacillin (bla_{OXA}), and Sulfhydryl (bla_{SHV}) variants. The screened *Escherichia coli* isolates were from human faecal, hand swabs, slurry, and fresh cattle dung samples.

identical to the class A *β*-lactamase, CTX-M-15 gene, extended-spectrum isolate with accession number KP268826 from the United Kingdom [20]. A representative sequence was deposited in the GenBank under accession number MZ314090.

3.4.2. Analysis of TEM Gene (bla_{TEM})

Twelve bla_{TEM} positive isolates were sequenced and compared with other published bla_{TEM} genes in *E. coli* strains from GenBank. Two different bla_{TEM} alleles were identified, 5 (42%) with the classical allele and 7 (58%) with a variant that differed from TEM-1 by two amino acids, in the single peptide region at positions 82 [blaTEM-1 (V82I)] and 182 [blaTEM-1 (A182V)] (Table 6). The variant showed 5 different SNPs with positions indicated at 18, 228, 244, 396, and 545 (Table 7). The TEM-1 classical isolates were 100% identical to the class A broad-spectrum β-lactamase TEM-1 with accession number MW646301 [20], and a representative sequence was deposited in the GenBank under accession number MZ314091. Meanwhile, the variant isolates were 100% identical to the class a broad-spectrum β-lactamase TEM-116 of an *Acinetobacter baumannii* isolate with accession number KU180704 [21]. The variant sequences are represented in the GenBank under the sequence accession number MZ314092.

Table 6. Amino acid substitutions of TEM-1 and TEM-116 β-lactamase.

β-lactamase	The residue (coding triplet) at amino acid	
	82	182
TEM-1	Val (GTT)	Ala (GCA)
TEM-116	Ile (ATT)	Val (GTA)

Table 7. Single nucleotide substitutions of bla_{TEM} gene found in twelve haplotypes of *Escherichia coli*.

Haplotype	bla_{TEM}				
	18	228	244	396	545
MW646301	T	T	G	T	C
5548 FCD	.	.	.	.	.
5534 HDS	.	.	.	.	.
5560 HFD	C	C	A	G	T
5577 HDS	C	C	A	G	T
5559 HFD	C	C	A	G	T
5577 HFD	C	C	A	G	T
5558 HDS	.	.	.	.	.
5556 HFD	C	C	A	G	T
5558 HFD	C	C	A	G	T
5542 CDS	C	C	A	G	T
5555 CDS	.	.	.	.	.
5555 HFD	.	.	.	.	.

The substitutional sites are numbered from the initiation codon of the TEM-1 gene. The length of the TEM-1 gene sequence was 861 bp. A complete gene sequence from the United Kingdom, accession number MW 646301, was used as the reference sequence.

3.5. Diversity of Clinical and Environmental *E. coli* Isolates

Most *E. coli* isolates did not cluster based on sample type or sampling location in the phylogenetic analysis. For instance, isolates recovered from faecal, hand swabs, and fresh cattle dung samples from farms in Githunguri were scattered across the cluster groups (Figure 2). There was also no homogeneity in antimicrobial resistance profiles in isolates recovered from the same region. However, two isolates from faecal and hand swab samples from Gatundu North **(cluster group VI)** had a similarity index of 100%, indicating that these isolates are genetically similar. However, the two isolates slightly varied in their antimicrobial resistance profiles, resulting from acquiring resistance genes via mobile genetic elements (Figure 6).

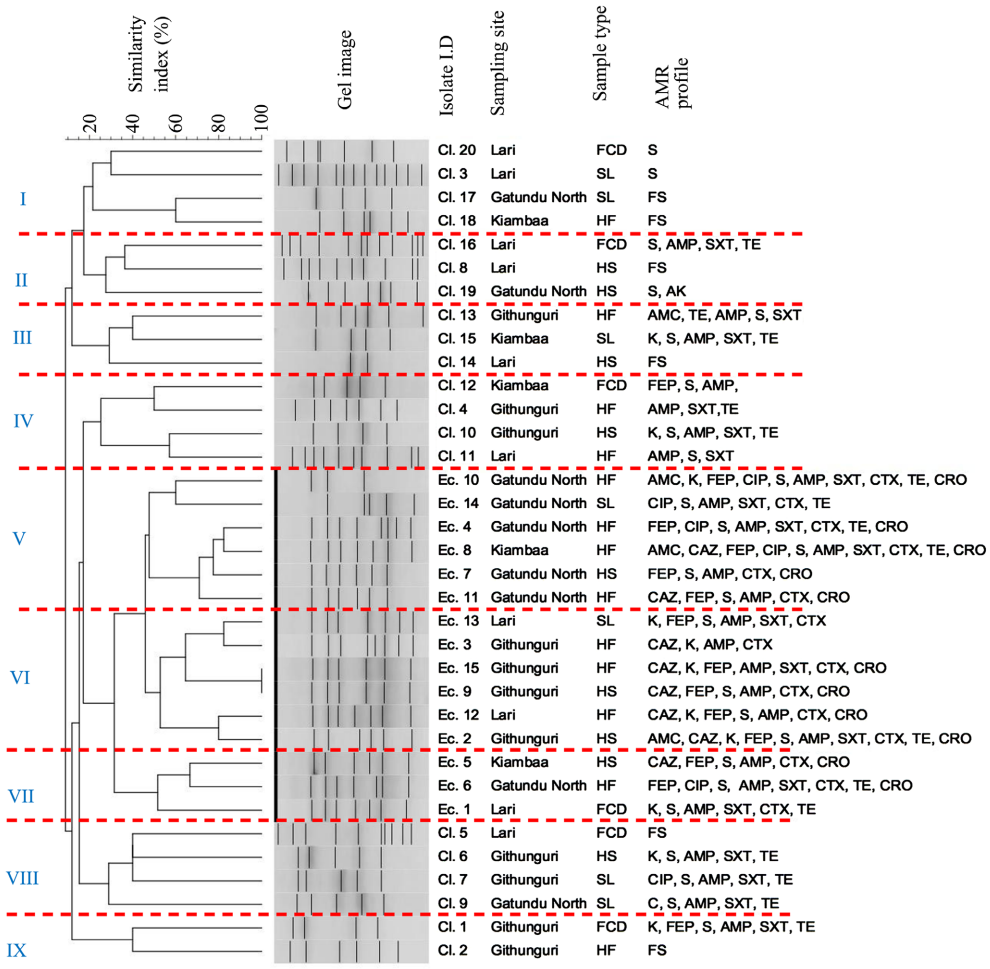

Figure 6. Clustering patterns of *Escherichia coli* isolates from human and non-human samples in Kiambu county. This dendrogram was generated from *Escherichia coli* isolates from human faecal (HF), hand swabs (HF), fresh cattle dung (FCD), and slurry (SL) samples. The samples were collected from farmers and farms in Kiambaa, Githunguri, Gatundu North, and Lari areas in the Central Kenya region. Description for various acronyms used in this dendrogram are; AMP: Ampicillin, AMC: Amoxicillin-clavulanic acid, CAZ: Ceftazidime, CIP: Ciprofloxacin, CTX: Cefotaxime, CRO: Ceftriaxone, FEP: Cefepime, SXT: Trimethoprim-sulfamethoxazole, S: Streptomycin, K: Kanamycin, TE: Tetracycline and FS: for isolates that were fully susceptible to tested antimicrobial agents.

4. Discussion

The menace of antimicrobial resistance (AMR) is an increasing global health concern and negatively impacts the fight against infectious diseases caused by bacterial pathogens [22]. Over the years, the emergence and gradual increase in antimicrobial resistance have been driven by misuse and over-use of antimicrobials in human healthcare and, more recently indiscriminate use in livestock and agriculture farming [23]. Although much is known about the drivers and burden of AMR on human health, research on livestock and agriculture has only recently started to gain traction.

To have an overview of the burden of AMR in livestock farming, the current study investigated *Escherichia coli* since it is a suitable indicator species for antimicrobial resistance [24]. A prevalence of 28% *E. coli* in untreated cattle slurry was recorded and was close to the 29% found in fresh cattle dung samples. These findings indicate that these bacterial isolates can survive for a long period, even beyond 90 days in harsh environmental conditions [25]. Cattle manure has traditionally been used as a rich source of essential nutrients for crop agriculture and is often deemed safer than chemical fertilisers that have a compounding effect on soil chemical composition after prolonged use [26]. However, manure is used in crop farming without prior treatment in most developing countries due to a lack of capital and technology. The use of untreated manure and animal slurry contaminates water and has been a source of enteric pathogens associated with foodborne infections [25]. The Food Safety Modernisation act recommends a 90 to 120-day waiting period from the time of untreated manure use and before crop harvest [25] [27]. This waiting period significantly reduces foodborne infections that may result from consuming vegetables or fruits contaminated with enteric of manure origin.

Cattle farming is prevalent in areas surrounding Nairobi County due to a broad market for milk. Urban cattle farming is also favourable due to direct contact with customers, removing the middlemen to increase profit margins. However, zero-grazing in animal sheds close to the homestead is an everyday phenomenon due to space limitations. Dung disposal is also a problem, and animal slurry in homestead compounds on farms with limited space is also very common [28]. Most farmers handling animal dung and slurry lack protective gear. Therefore, this poses a risk of animal-human enteric bacterial cross-contamination, which can eventually cause zoonotic diseases. Although the origin of *E. coli* isolates from hands swabs was not determined in this study, some of these bacteria may have emanated from handling the animal dung, slurry, or even human faecal, as no homogenous clustering patterns per sample type were observed. Non-bloody diarrhea has been associated with pathogenic strains such as Shiga-toxin *Escherichia coli* (STEC) of animal origin [29]. Caution, therefore, needs to be taken to avoid contamination of animal products such as meat and milk with these bacterial strains.

In this study, most of the MDR *E. coli* isolates (n = 40) were resistant to three antimicrobial classes, followed by 37 (13%) resistant to four antimicrobial classes.

Three (1%) MDR isolates were resistant to eight antimicrobial classes. These findings agree with those reported in an earlier study [30], which found that 36.7% of MDR isolates recovered from selected sites in Kenya. There is a great need to investigate the presence of mutations in the general outer membrane porins that drugs use for entry, assess the increased expression of efflux systems, integrons, and plasmids associated with AMR, and mutations in other genes encoding resistance enzymes.

The antimicrobial resistance profiles analysis by sample type revealed that non-susceptible isolates from human faecal were more predominant than those from animal samples, which is consistent with findings from a previous study in Kenya [31]. Although isolates from animal samples have relatively minimal AMR compared to human samples, AMR in livestock samples has increased in Africa and other parts of the globe [32]. We found that most isolates from cattle dung and slurry are susceptible to most antimicrobial agents. Resistance towards tetracycline, aminoglycosides, and sulfonamides ranged between 2% - 34%, which is lower than the 37% - 58% reported earlier [31]. Resistance determinants against these antimicrobials are often situated in the same plasmid together with genes mediating resistance to heavy metals and some antibiotics such as streptomycin [33]. The use of household disinfectants and acaricides is reported to select multi-resistant mutants either by a mutation on the target gene or in the regulatory *mar* system, providing pleiotropic resistance to disinfectants and multiple structurally unrelated antibiotics and oxidative stress agents [34]. According to this report, other factors other than the use of veterinary and human medicines could be leading to the stability of antibiotic resistance in dairy farms. In the current study, resistance towards cephalosporins and amoxicillin-clavulanic acids ranged between 2% - 23%, higher than the 2% - 3% in the previous study [31]. The difference in AMR profiles could result from variation in antimicrobial use in both humans and livestock in different sub-counties in Kiambu.

In this study, all *E. coli* isolates producing $bla_{CTX\text{-}M}$ enzymes also had the bla_{TEM} gene. However, these isolates showed no relationship in their resistance profiles. This phenomenon of co-carriage of the ESBL gene has previously been reported in a similar study [30]. Among the *E. coli* isolates resistant to 3rd generation cephalosporins and phenotypically positive for ESBL production, 3 (17%) isolates were $bla_{CTX\text{-}M}$ and bla_{TEM} negative. These results suggest that the isolates could have mutations in the promoter region of the multi-resistance gene cassette or due to point mutations within the primer regions [30] [35]. There is a risk of spreading and exchanging the *bla* genes between animal, environmental, and clinical samples. A previous study showed that the increased prevalence of the CTX-M-15 *E. coli* in some cattle groups and immediate environments was related to husbandry, general hygiene, and antimicrobial usage [36]. Although not investigated in this study, the presence of ESBL mediated resistance could result from using cephalosporins to treat mastitic and diarrhoeal diseases such as scour in calves and co-selection through using other classes of antibiotics that can select for ESBLs. Another study reported a similar co-acquisition of resistance genes in

E. coli strains isolated from a dairy farm [37].

This study found two bla_{TEM} variants (TEM-1 & TEM-116) and $bla_{CTX\text{-}M\text{-}15}$ genes circulating within Kiambu county. The TEM-variant differed from the TEM-1 gene by two-point mutations at amino acids, substituting the valine residue at position 82 for isoleucine (Val82Ile) and alanine at position 182 for valine (Ala182Val). These two mutations were encountered in TEM-116 β-lactamase. In addition, three nucleotides of this variant were silent point mutations: T-to-C exchange at nucleotides 18 and 228, and a T-to-G at nucleotide 396. These amino acid mutations, which were not previously detected in other TEM type variants, may be involved in the TEM-116 enzyme's high affinity for ceftazidime, cefotaxime, and other extended-spectrum β-lactams [38]. TEM-1 β-lactamase is found at high frequencies in antimicrobial-resistant bacteria (ARB). While TEM-1 has a spectrum limited to penicillins and early cephalosporins, it has given rise to more than 180 descendent alleles, such as TEM-116. These alleles confer resistance to most modern β-lactam antibiotics. We believe this is the first report on the presence of TEM-116 in a dairy farm set-up in Africa. Therefore, these findings highlight the remarkable possibility of circulating TEM-116 in Kenya and her neighbouring countries. Whether isolates of *E. coli* in the farm produce other ESBLs and whether the TEM-types are the direct evolutionary consequences of the original TEM-1 is worthy of further study.

The (GTG)5 PCR has the highest discriminatory power of all the Rep-PCR methods [17]. This study's fingerprinting technique (GTG)5 was used for its ability to intraspecies differentiation of *E. coli* genomes [39] and showed that the isolates from cattle handlers, immediate environment, and cattle dung had no absolute distinction and were dispersed across all the cluster groups. Previous studies of *E. coli* from cattle feces and farm environments have shown that clonally related strains harbor the same ESBL genes seen in cultivated soil embedded with manure a year before [40]. The genotyping method employed in this study showed genomic diversity in the *E. coli* isolates with no evidence of clonal lines. Moreover, identical genotypes had different antibiotic resistance profiles. Both of these findings suggest the presence of mobile resistance determinants in the population. Since the study used low-resolution fingerprinting techniques, there is a need to access the virulence factors and genetic features of such isolates using high-resolution methods such as whole-genome sequencing.

5. Conclusion

The data from this study draws a clear picture of high antibiotic resistance patterns towards sulfamethoxazole/trimethoprim, tetracycline, ampicillin, and other β-lactams. This study also showed that meropenem and kanamycin antimicrobials are still potent against human and livestock bacterial isolates. Although isolates from human samples have the highest prevalent AMR profiles, isolates from animal samples have relatively high resistance. There is, therefore, a poten-

tial for MDR bacterial strains to ensue from livestock reservoirs. The high prevalence of MDR phenotypes and co-carriage of resistance genes calls for further studies on the molecular basis of resistance amongst these isolates and their mobility. Although cattle dung is a cheap source of organic manure for crop farming, the risk of untreated manure should not be overlooked.

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Authors' Contributions

D. Waithiru was involved in the conception, design, data collection, and analysis and drafted the manuscript. J. Mwaniki, J. Maingi, and J. Kiiru provided expert advice in designing, implementing, and coordinating the study. E. Mulinge, J. Maina, and B. Ngugi assisted with the data collection and analysis. All authors contributed to the writing of the manuscript and approved the submission of the final manuscript.

Ethical Consideration

This study was approved by the KEMRI IRB (SERU); KEMRI/SERU/CMR/P00127/3927 before the sample collection was started. In addition, permission to conduct this study was obtained from the National Commission for Science, Technology, and Innovation (NACOSTI): NACOSTI/P/22/15775 and Kiambu county public health and veterinary services.

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Conflicts of Interest

There is no conflict of interest.

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