BAGHDAD JOURNAL OF BIOCHEMISTRY AND APPLIED BIOLOGICAL SCIENCES
2024, VOL. 5, NO. 1, 36-58, e-ISSN: 2706-9915, p-ISSN: 2706-9907
https://doi.org/10.47419/bjbabs.v5i01.265

ORIGINAL ARTICLE

Occurrence and antimicrobial susceptibility of Enterobacteriaceae from public transport in Dar es Salaam, Tanzania

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ABSTRACT

This study aimed to investigate the occurrence and antimicrobial susceptibility of clinically relevant Enterobacteriaceae from public transport in Dar es Salaam, Tanzania. A total of 100 pooled swab samples were collected from public buses in Dar es Salaam from January 2023 to April 2023. Enterobacteriaceae (Escherichia coli, Enterobacter spp., and Klebsiella spp.) were isolated and identified using standard microbiological techniques. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method. PCR amplification was carried out to detect the presence of antibiotic resistance genes. Furthermore, the 16S rRNA gene of isolates that exhibited phenotypic resistance to all tested antibiotics was Sanger sequenced and a phylogenetic tree was constructed to evaluate their genetic diversity. There was difference in abundance among Enterobacteriaceae in the study. The Klebsiella spp. (68) was the most abundant, followed by Escherichia coli (48) and the least abundant was Enterobacter spp., where 15 isolates observed. Similarly, a high AMR profile was observed against ampicillin (100%) followed by Amoxicillin (97%). Also, isolates resistant to multiple drugs (MDR) were common and very prevalent, where out of 131 isolates, 129 (98%) were MDR. Furthermore, the findings showed a strong positive correlation between phenotypic resistance and the presence of resistance genes (r_s= 0.66, P<0.05). Furthermore, the molecular identification confirmed the PDR isolates were distributed across three genera: Escherichia, Enterobacter, and Klebsiella. Also, the phylogenetic analysis indicated the PDR isolates interspersed with reference sequences within their respective genera. The present findings highlighted the high abundance and prevalence of AMR clinically important Enterobacteriaceae and underscore the importance of instituting surveillance programs designed to combat AMR focusing on public transport in developing countries.

Keywords Antimicrobial resistance, Enterobacteriaceae, multi-drug resistance

Received 2023-11-27
Revised 2023-12-22
Accepted 2023-12-25

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DOI https://doi.org/10.47419/bjbabs.v5i01.265

Pages: 36-58

INTRODUCTION

An endeavor to improve global human health and development is compromised by the threat of antimicrobial resistance [1]. Antimicrobial resistance (AMR) is among the leading top ten challenges to global health and development It was estimated that out of the 4.95 million total deaths in 2019, 1.27 million were directly related to a drug-resistant infection globally [2]. The health burden attributed to AMR is even higher than malaria and HIV/AIDS combined[2].

The immense burden of AMR is highly pronounced in developing countries due to extensive poverty acerbated by mishandling of antibiotics [3]. Taken as an example, the estimate of multidrug-resistant (MDR) bacteria was about 25% following analysis of samples collected from clinical settings in Tanzania [4]. Astonishingly, the same trend (the proportion of 66%)) was observed following the assessment of samples collected from wounds of patients at the Muhimbili National Referral Hospital in Tanzania. The AMR was about 66% against at least three antimicrobial types (the class cephalosporin, aminoglycosides, tetracycline, fluroquinolones, folate pathway inhibitors, phenicol, macrolides and clinolamides) [4]. The information on the prevalence and pervasive nature of AMR corroborates on the conjecture that paucity of concerted effort to counter this problem may led to global calamity.

Antimicrobial resistance arises when bacteria, viruses, fungi, and parasites adapt and thrive in the antimicrobial agents that were once effective against them [5, 6]. The development of AMR occurs through several mechanisms, such as antibiotic modification, effiux pump, target modification , and target replacement or target bypass [7]. One prevalent mechanism of bacterial resistance involves the action of β-lactamase enzymes, which is the most used mechanism in bacteria. These enzymes hydrolyze the amide bond in the β -lactam ring, rendering the antibiotics inactive. These enzymes are found in the periplasmic space of gram-negative bacteria such as Enterobacteriaceae., acting as barrier that prevent the antibiotic reaching the penicillin binding proteins (PBPs), thus inactivates the drug before it can exert its effect[6]. Furthermore, microbes may acquire AMR properties naturally over time through genetic mutation[8]. Other contributing factors for AMR acquisition by microbes include poor management of antibiotics like misuse and overuse of antimicrobials [7].

Various hotspots for the transmission of AMR pathogens among the communities have been identified[9]. However, one often neglected vehicle for spread of AMR bacteria is public transport, particularly in poor communities where there is overcrowding of people and poor hygiene practices [10]. Public transport like buses, and community rapid transports provide an environment where transmission of pathogens between individuals can easily occur[11]. For instance, in a study conducted in Ethiopia, 100% of E. coli and Enterobacter spp. isolates shown ampicillin resistance, while 34% of E. coli and Enterobacter spp. isolates demonstrated chloramphenicol resistance among the bacteria isolated from public bus swab samples [12]. Despite the well-known role of public transport in the transmission of AMR strains among people, there are lack of compressive surveillance programs designed to detect and map AMR transmission hotspots including public transport in developing

countries like Tanzania. Therefore, to layout a foundation for AMR surveillance programs in public transport in Tanzania, the present study was aimed to investigate occurrence of AMR in Enterobacteriaceae of clinically relevant isolated from public transports in Dar es Salaam, Tanzania.

MATERIALS AND METHODS

SAMPLING LOCATION

The sampling sites involved in the present study are depicted on a map of Dar es Salaam (Figure 1) showing bus stops that were used as the sampling points in this study and their possible road routes.

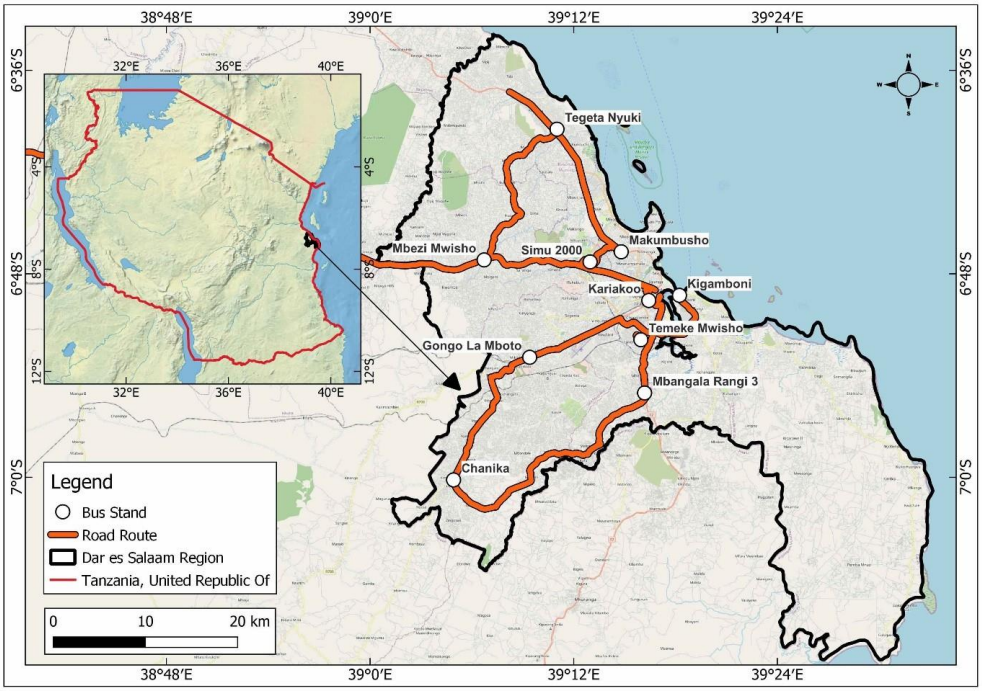

Figure 1 A map of Dar es Salaam showing bus stations that were used as sampling points in the study

SAMPLE COLLECTION

From January 2023 to April 2023, Samples were collected from ten sampling points (Mbagala, Gongo la Mboto, Tegeta, Kigamboni, Buza, Tandika, Muhimbili, Makumbusho, Mbezi and Chanika bus stops) as shown on Figure 1, which were purposively selected based on the population and those routes with high ridership. For each sampling point site, ten

buses were randomly selected and Swab samples were collected while the bus completed the trip (at the end of the journey). From each bus at least five cotton swabs were taken and pooled to make one sample, and therefore, from each sampling point ten samples were collected, and therefore about 100 samples were collected in total.

The sample collection media was prepared as previously defined by Kahsay et al. (2019), with minor modifications. Briefly, a 5 ml test tube containing 2 ml peptone water and normal saline and an application cotton swab was autoclaved at 121˚C for 15 minutes. During sample collection and transportation, sample collection materials (peptone water, normal saline and cotton swabs) were placed in cool box containing ice-packs to maintain cold chain. Normal saline was used to moisture sterile cotton swabs before rubbing to the frequently touched surface of buses, and then the rubbed swabs were placed ascetically in sterile tube containing peptone water (enrichment medium). Immediately after sampling, the samples were transferred in a cooler box to the University of Dar es Salaam (UDSM), Department of molecular biology and biotechnology (MBB), for microbiological analysis.

ISOLATION AND IDENTIFICATION OF SELECTED ENTEROBACTERIACEAE OF CLINICAL RELEVANCE

The Enterobacteriaceae under the present study were isolated following procedures described in previous studies [12–14]. However, minor modifications were implemented and therefore, the briefly description of the protocol is provided. The first step was initial growth of bacterial on nutrient agar for overnight, and then the overnight cultured were streaked on a selective medium a MacConkey Agar (MAC). Colonies characteristic (pink or ed in color) of E. coli, Klebsiella spp., and Enterobacter spp on MAC agar were sub-cultured on a differential media an Eosin Methylene Blue (EMB) agar. Pure cultures were prepared by streaking few cells from a single colony on a nutrient agar plate. All incubations were at 37 ˚C for 24 hours.

The identification of isolates was based on colonies morphology, Gram reaction, and biochemical tests including indole synthesis, citrate utilization, motility test, Methyl Red (MR), and the Voges Proskauer (VP) test (Table 2). The isolates were preserved at -20˚C in nutrient broth (NB) containing 15% glycerol.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antibiotic susceptibility testing was done using the Kirby-Bauer disk diffusion method on Muller-Hinton agar (MHA) in line with the 30^{th} edition Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. Standard panel of commonly prescribed antibiotics for human illness and diseases caused by Enterobacteriaceae family were used to evaluate antibiotic resistance profiles of the isolates.

The isolated organisms that had been stored in glycerol stocks were revived in nutritional broth and cultured for 24 hours at 37˚C. The culture was used to produce a bacterial solution that met the 0.5 McFarland criteria using sterile swab sticks (1.5×10^8 CFU/ml), which was then evenly inoculated onto MHA and left to dry for 10 minutes. The flesh prepared discs containing the following antibiotics: Amoxicillin (AMC,10μg), Ampicillin (AMP, 10μg), Ceftriaxone (CEF, 30μg), Azithromycin (AZI, 15μg), Cephalexin (CEP,30μg), Ciprofloxacin (CIP, 5μg), Norfloxacin (NOR, 10μg), Trimethoprim/Sulfamethoxazole (TRI/SUL,25μg), Chloramphenicol (CHL, 30 μg), Gentamicin (GM,10 μg), Tetracycline (TET, 30 μg) and Erythromycin (ERY, 15 μg) were aseptically placed onto the surfaces of the sensitivity agar plates with a sterile forceps and gently pressed to ensure even contact. The E. coli ATCC 25922 was used as the quality control strain throughout the antimicrobial susceptibility testing. After incubation at 37 ˚C for 24 hours, the diameters of growth inhibition were measured in millimeters and classified as resistant (R), intermediate (I) and susceptible (S). Isolates resistant to at least one antibiotic from three or more antimicrobial classes, all agents in all antibiotic classes, and at least one agent in all but two or fewer antibiotic groups were classified as multidrug resistant (MDR), Pan drug-resistant (PDR), and extensively drug-resistant (XDR), respectively[16].

GENOMIC DNA EXTRACTION

Genomic DNA (gDNA) extraction of Enterobacteriaceae was done by CTAB protocol as previously described[17]. The concentration and purity of DNA was determined using NanoDrop^{TM} One^{C} (Thermo Fisher Scientific, USA) through 260/280 nm absorbance ratio and DNA visualization was done by using 1% agarose gel electrophoresis.

GENOTYPING OF AMR ISOLATES AND RESISTANCE GENE DETECTION

Genomic DNA of all isolates subjected for PCR amplification of 16S rRNA using universal primers to determine their identity. Furthermore, the genomic DNA of isolates that exhibited resistance by phenotypic approach were subjected to a PCR to detect the presence of commonly reported antibiotic resistant genes that have been associated with a particular resistance. The evaluated genes include; tetracycline (tet(A)), beta-lactams (blaCTX-M and blaTEM), gentamicin (aac ^{3}-IV), chloramphenicol (catAI), and trimethoprim/sulfamethazol (dfrAI, and sulI). The primer-pair sequences that were used for PCR, their annealing temperature and PCR amplicon sizes are shown in Table 1.

Each PCR reaction was carried out in a final volume of 25 μL, which contained 9.5 μL of nuclease-free water (Water for Molecular Biology Bioconcept 3-07F04-I), 12.5 μL of 2X OneTaq Master Mix (New England Biolabs), 2 μL of normalized DNA to the concentration of 200 ng/μL as template, and 1 μL (10 pmol) of each primer set. For the amplification of

Table 1. Nucleotide sequences of primers for the detection of 16S rRNA, and resistance genes of Enterobacteriaceae.

Gene Target	Primer Sequence (5'-3')	Expected Amplicon (bp)	Annealing Temp (˚C)	
16S rRNA	(27F) AGAGTTTGATCCTGGCTCAG (1495R) CTACGGCTACCTTGTTACGA	1500	55	18
Aac(3)-IV	(F) CTTCAGGATGGCAAGTTGGT (R) TCATCTCGTTCTCCGCTCAT	286	55	19
Sul1	(F) TTCGGCATTCTGAATCTCAC (R) ATGATCTAACCCTCGGTCTC	822	47	19
catA1	(F)AGTTGCTCAATGTACCTATAACC (R)TTGTAATTCATTAAGCATTCTGCC	547	55	19
Tet(A)	(F) GGTTCACTCGAACGACGTCA (R) CTGTCCGACAAGTTGCATGA	577	57	19
dfrA1	(F) GGAGTGCCAAAGGTGAACAGC (R)GAGGCGAAGTCTTGGGTAAAAAC	367	45	19
bla$_{TEM}$	(F) ACATGGGGGATCATGTAACT (R) GACAGTTACAATGCTTACT	421	52	20
BlaCTX-M	(F3) GACGATGTCACTGGCTGAGC (R2) AGCCGCCGACGCTAATACA	499	55	21

16S rRNA, PCR conditions were as follows: initial denaturation at 94°C for 30 seconds, 35 cycles of denaturation at 94 °C for 30 seconds, annealing temperature at 55 °C, extension at 68 °C for 1 minute, and final extension at 68 °C. For the amplification of resistance genes, PCR conditions were as follows: in brief, consisted of an initial denaturation step at 95 ˚C for 10 minutes, 35 cycles of denaturation at 95 ˚C for 30 seconds, annealing at a temperature appropriate for each primer used for 30 seconds, extension for 1 minute at 72 ˚C, and a final extension at 72 ˚C for 10 minutes. PCR products were confirmed by electrophoresis in 1.8% agarose gels stained with safe view^{TM} classic dye, Quick-load 1kb plus DNA ladder (New England Biolabs, Inc) was used as a molecular marker, and were visualized in the Gel.LUMINAX Gel Documentation System - BioZEN Labs (Nagar, India).

SEQUENCING OF 16S RRNA AND BIOINFORMATICS ANALYSIS

The 16S rRNA amplicons of isolates that exhibited phenotypic antimicrobial resistance to all antibiotics tested, were sent to Macrogen (Amsterdam, Netherlands) for Sanger sequencing in order to identify bacteria. Quality control for the raw sequences were done using CLC Genomic Workbench V.8. To identify the bacterial species, a BLAST search tool on the National Center for Biotechnology Information (NCBI) database was employed.

DATA ANALYSIS

Descriptive and inferential statistics was performed using R software (4.3.0 version). Shapiro wilk test was used to determine the normality. The data on abundance of Enterobacteriaceae were normally distributed, and therefore one-way analysis of variance (ANOVA) was conducted to ascertain inferential statistics on bacterial counts at different locations, and pair-wise comparison of the same was done by Tukey's HSD test. Furthermore, the difference in the levels (percentage) of antimicrobial susceptibility among isolates was evaluated by Kruskal Wallis test. In addition, correlation of antimicrobial susceptibility results and resistance genes was determined using Spearman rank correlation test and strength of the association was interpreted as previous described[22]. The $P \leq 0.05$ levels was used as significance difference. Furthermore, in order to establish the evolutionary relatedness among the isolates, relevant reference sequences of antibiotic-resistant strains were retrieved from the GenBank database Then, sequences generated in this study were aligned along with those downloaded from GenBank using MUSCLE algorithm. After the alignment process, a phylogenetic tree was constructed using MEGA 11.0 software as previously described[23].

RESULTS

IDENTIFICATION OF SELECTED ENTEROBACTERIACEAE OF CLINICAL RELEVANCE

A total of 100 pooled swab samples taken from frequently touched surfaces of various buses at various bus stops in Dar es Salaam were tested for the presence of Enterobacteriaceae of clinical importance (Klebsiella spp., Enterobacter spp., and E. coli) as portrayed in Table 2.

Table 2. Microscopy and biochemical characteristics of clinically relevant Enterobacteriaceae (E. coli, Klebsiella spp., and Enterobacter spp.) in the study

Characterization	Isolates		
	E. coli	**Klebsiella spp.**	**Enterobacter spp.**
MCA	Pink or red, and non mucoid colonies	Large, pink or red, and mucoid colonies	Pink or red, and mucoid colonies
EMB	Non mucoid, dark blue-black colonies with metallic green sheen	Large, pink, dark-centered, mucoid colonies	Large, Pink, mucoid colonies
Microscopy	Pink and rod shaped	Pink and rod shaped	Pink and rod shaped
Indole	+	-	-
MR	+	-	-
VP	-	+	+
Citrate	-	+	+
Motility	+	-	+

MCA = MacConkey agar; EMB = Eosin Methylene Blue agar; MR = Methyl Red; VP = Voges Proskauer; + = Positive; - = Negative.

ABUNDANCE OF ENTEROBACTERIACEAE OF CLINICAL RELEVANCE

Samples were examined for the presence of bacteria of clinically importance belonging to the Enterobacteriaceae family including E. coli, Klebsiella spp., and Enterobacter spp. Surprisingly, 98 out of 100 samples (98%) tested positive for the bacteria type involved in the present study. A total of 131 Enterobacteriaceae were isolated. Notably, the abundance of E. coli and Klebsiella spp. was found to be significantly higher than that of Enterobacter spp., ($P<0.05$) as shown in Table 3. However, the study did not find any significant influence of location on the abundance of these bacterial isolates ($P>0.05$).

Table 3. The abundance of Enterobacteriaceae isolated in the study

Sampling Location	Species			Total
	E. coli	Klebsiella spp	Enterobacter spp	
Sm 2000	4	8	0	12
Makumbusho	5	4	0	9
Tegeta	3	5	0	8
Mbezi	4	11	2	17
K/Koo	5	8	0	13
Mbagala	6	9	3	18
Temeke	7	2	6	15
Kigamboni	5	7	1	13
G/Mboto	6	6	2	14
Chanika	3	8	1	12
Total	48(37%)	68(52%)	15(11%)	131
Mean±SEM	4.0 ± 0.15^{a}	6.8 ± 0.83^{a}	1.5 ± 0.60^{b}	

Means of the same row bearing different superscript are significantly different (Tukey's HSD, $P<0.05$), Sm 2000 = Mawasiliano bus station, K/koo = Kariakoo bus station, G/mboto = Gongo la mboto bus station.

ANTIMICROBIAL PROFILE OF ENTEROBACTERIACEAE OF CLINICAL RELEVANCE

Antibiotic sensitivity of all isolates against 12 selected antibiotics was done using the Kirby-Bauer disk diffusion method. The overall resistance profile expressed in percentage showed high resistance to Ampicillin and Amoxicillin, which had 100% and 97% resistance, respectively. Norfloxacin, Azithromycin, and Ceftriaxone were the most susceptible antibiotics, as they showed 24%, 22%, and 21% resistance, respectively as shown in Figure 2.

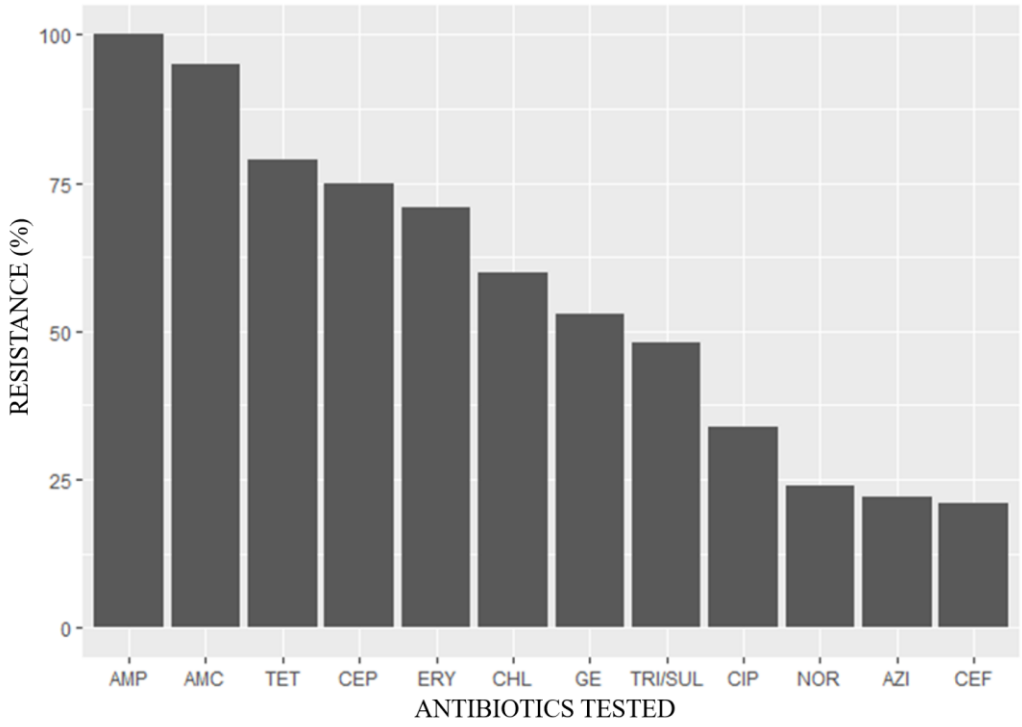

Figure 2 Antimicrobial profile of Enterobacteriaceae against tested antibiotics. TET = Tetracycline, GE = Gentamicin, AMP = Ampicillin, ERY = Erythromycin, CIP = Ciprofloxacin, AZI = Azithromycin, CEP = Cephalexin, AMC = Amoxicillin, NOR = Norfloxacin, CEF = Ceftriaxone, CHL = Chloramphenicol, and TRI/SUL = Trimethoprim/Sulfamethoxazole.

The antimicrobial profile of E. coli against tested antibiotics showed high resistance to Ampicillin (100%) and Amoxicillin (94%), and low resistance was observed for Azithromycin and Ceftriaxone, which showed 21% and 23% resistance, respectively. The antibiotic susceptibility of Klebsiella isolates was examined, and they were found to be highly resistant to Ampicillin (100%) and Amoxicillin (94%). Ceftriaxone and Norfloxacin showed the lowest rates of resistance to Klebsiella, at 19% and 16%, respectively. The antibiotic susceptibility of Enterobacter isolates was also examined, 100% of the Enterobacter isolates that were tested were resistant to Ampicillin and Amoxicillin. Only 1 (7%), and 4 (27%) of the examined Enterobacter isolates were Ceftriaxone and Azithromycin resistant. The finding did not show the influence of species isolated on the antimicrobial resistance profile against 12 tested antibiotics ($P>0.05$); however, there was a statistical difference in the percentage resistance among antimicrobials tested ($p<0.05$) as shown in Table 4.

(n) = Number of isolates, TET = Tetracycline, GE = Gentamicin, AMP = Ampicillin, ERY = Erythromycin, CIP = Ciprofloxacin, AZI = Azithromycin, CEP = Cephalexin, AMC = Amoxicillin, NOR = Norfloxacin, CEF = Ceftriaxone, CHL = Chloramphenicol, and TXS = Trimethoprim/Sulfamethoxazole.

Table 4. Antimicrobial resistance profile of Enterobacteriaceae against 12 antibiotics expressed in percentage

Isolates	Total (n)	Test Drug											
		TET	AMP	ERY	CIP	AZI	CEP	AMC	GE	NOR	CEF	CHL	TXS
E. coli	48	75	100	79	33	21	77	94	54	33	23	65	46
Klebsiella spp	68	82	100	66	29	26	76	96	49	16	19	53	46
Enterobacter spp	15	73	100	67	53	7	60	100	73	33	27	73	67

The antimicrobial resistance profile of Enterobacteriaceae from different sampling locations against tested antibiotics was examined. The finding did not show the influence of the sampling location on the antimicrobial resistance profile against 12 tested antibiotics ($P>0.05$); however, there was a unique observation on the antimicrobial resistance profile of isolates from Tegeta bus station and Kigamboni bus station, in which all isolates were susceptible to Norfloxacin and Ceftriaxone, respectively as shown in Table 5.

Table 5. Antimicrobial resistance profile of Enterobacteriaceae with respect to sampling location against 12 antibiotics expressed in percentage.

Sampling Location	Total (n)	TEST DRUG											
		TET	AMP	ERY	CIP	AZI	CEP	AMC	GE	NOR	CEF	CHL	TRI/-SUL
Simu 2000	12	75	100	92	67	25	83	92	42	42	8	58	50
M/m-busho	9	89	100	89	56	11	67	100	22	11	22	56	78
Tegeta	8	88	100	63	38	13	88	100	13	0	13	100	13
Mbezi	17	88	100	65	6	18	82	100	53	6	12	59	53
Kariakoo	13	62	100	92	46	38	69	85	38	46	69	69	46
Mbagala	18	78	100	83	50	39	67	100	94	33	28	83	67
Temeke	15	80	100	67	40	7	60	100	87	47	33	73	67
Kigam-boni	13	85	100	100	8	8	62	92	15	8	0	31	8
G/mboto	14	79	100	50	29	21	93	93	29	7	14	50	21
Chanika	12	67	100	67	8	33	92	92	33	33	8	17	83

(n) = Number of isolates, TET = Tetracycline, GE = Gentamicin, AMP = Ampicillin, ERY = Erythromycin, CIP = Ciprofloxacin, AZI = Azithromycin, CEP = Cephalexin, AMC = Amoxicillin, NOR = Norfloxacin, CEF = Ceftriaxone, CHL = Chloramphenicol, and TRI/SUL = Trimethoprim/Sulfamethoxazole, Simu 2000 = Mawasiliano bus station, M/m-busho = Makumbusho bus station, G/mboto = Gongo la mboto bus station.

Furthermore, the 12 tested antibiotics were put in eight groups based on standard antibiotics categories to establish the prevalence of MDR isolates. Overall, the present findings showed a high prevalence of MDR isolates, in which out of 131 isolates of Enterobacteriaceae, 129 (98%) were MDR, 114 (87%) and 15 (12%) were XDR and PDR, respectively. Specifically, for MDR, there was no difference ($P>0.05$) among isolates types (E. coli, Kleb-

siella spp., and Enterobacter spp.), and also there was similar observation for XDR where no difference among isolate types. In contrast, PDR of Enterobacter spp. was significantly higher than E. coli and Klebsiella (Table 6).

Table 6. Prevalence of multidrug resistance of Enterobacteriaceae against 8 antimicrobial categories

Isolates	(n)	R0	R1	R2	R3	R4	R5	R6	R7	R8	MDR (%)	XDR (%)	PDR (%)
E. coli	48	0	0	0	8	2	18	7	8	5	48(100)	43(89)	5(10)
Klebsiella spp.	68	0	0	2	5	16	10	21	9	5	66(97)	61(89)	5(8)
Enterobacter spp.	15	0	0	0	1	5	1	1	2	5	15(100)	10(66)	5(33)
Total	131	0	0	2	14	23	29	29	19	15	129(98)	114(87)	15(12)

(n) = Number of isolates, R0 = no antibiotic resistance; R1 = resistant to one antimicrobial category; R2 = resistant to two antimicrobial categories; R3 = resistant to three antimicrobial categories; R4 = resistant to four antimicrobial categories; R5 = resistant to five antimicrobial categories; R6 = resistant to six antimicrobial categories; R7 = resistant to seven antimicrobial categories; R8 = resistant to eight antimicrobial categories; MDR = multidrug resistance; XDR = extensively drug-resistance; PDR = pan drug-resistance

GENOTYPIC AND PHENOTYPIC AGREEMENT AMONG ENTEROBACTERIACEAE

Overall, the findings showed a strong positive correlation (r_s= 0.66, P<0.05) between resistance phenotype and presence of resistance gene(s). However, in several isolates that showed phenotypic resistance to specific antibiotics, the evaluated resistance genes were not detected (Table 7). For instance, Gentamicin and Aac(3)-IV resistance gene exhibited 0% concordance in all isolates, while Trimethoprim/Sulfamethoxazole and dfrA1 resistance gene had 100% phenotypic/genotypic concordance (Table 7).

MOLECULAR IDENTIFICATION AND RELATEDNESS OF PAN-DRUG RESISTANT ENTEROBACTERIACEAE

Twelve isolates that exhibited phenotypic resistance to all tested antibiotics were Sanger sequenced for the 16S rRNA gene using universal primers. Following submission to NCBI database, the sequences were assigned with unique accession numbers as shown in Table 8. As it was expected, moleculr identification process by DNA sequencing confirmed that the 12 isolates were distributed across three genera: Escherichia, Enterobacter, and Klebsiella. Furthermore, the identities of the 16S rRNA gene sequences for the isolates ranged from 93% to 99% indicating high levels of similarity to known reference sequences (Table 8). The genus Escherichia isolates comprised E. coli, the genus Enterobacter isolates included

Table 7. Genotypic-phenotypic resistance agreement among Enterobacteriaceae of clinical importance from public transport.

Antibiotic Class	Antibiotic	Antibiotic Resistance Characterized	E. coli (48)	Klebsiella spp. (68)	Enterobacter spp. (15)	Total (131)
B-Lactams (Penicillin, Cephalosporins)	Ampicillin, Amoxicillin, Cephalexin, Ceftriaxone	Phenotypic Resistance	48	68	15	131
		Genotypic Resistance: blaTEM	38	55	12	105
		Genotypic/Phenotypic Agreement	79%	81%	80%	80%
		Genotypic Resistance: blaCTX-M	13	19	3	35
		Genotypic/Phenotypic Agreement	27%	28%	25%	27%
Tetracyclines	Tetracycline	Phenotypic Resistance	36	56	11	103
		Genotypic Resistance: Tet(A)	14	29	6	49
		Genotypic/Phenotypic Agreement	39%	52%	55%	48%
Aminoglycosides	Gentamicin	Phenotypic Resistance	26	33	11	70
		Genotypic Resistance: Aac(3)-IV	0	0	0	0
		Genotypic/Phenotypic Agreement	0%	0%	0%	0%
Phenols	Chloramphenicol	Phenotypic Resistance	31	36	11	78
		Genotypic Resistance: catA1	11	16	2	29
		Genotypic/Phenotypic Agreement	35%	44%	18%	37%
Folate Pathway Inhibitor	Trimethoprim/ Sulfamethoxazole	Phenotypic Resistance	22	31	10	63
		Genotypic Resistance: Sul1	9	15	5	29
		Genotypic/Phenotypic Agreement	41%	48%	50%	46%
		Genotypic Resistance: dfrA1	22	31	10	63
		Genotypic/Phenotypic Agreement	100%	100%	100%	100%

E. cloacae, and the genus Klebsiella isolates included K. pneumoniae and K. oxytoca with their specific strains as shown in Table 8.

AC = Accession number

In order to establish the evolutionary relatedness among the isolates from this study and other reported isolates. The phylogenetic relatedness of PDR isolates is displayed in Figure 3. The phylogenetic analysis indicated that the pan-drug resistant isolates were interspersed with reference sequences within their respective genera. The analysis also revealed distinct clustering patterns based on the genus and species of the isolates (Figure 3).

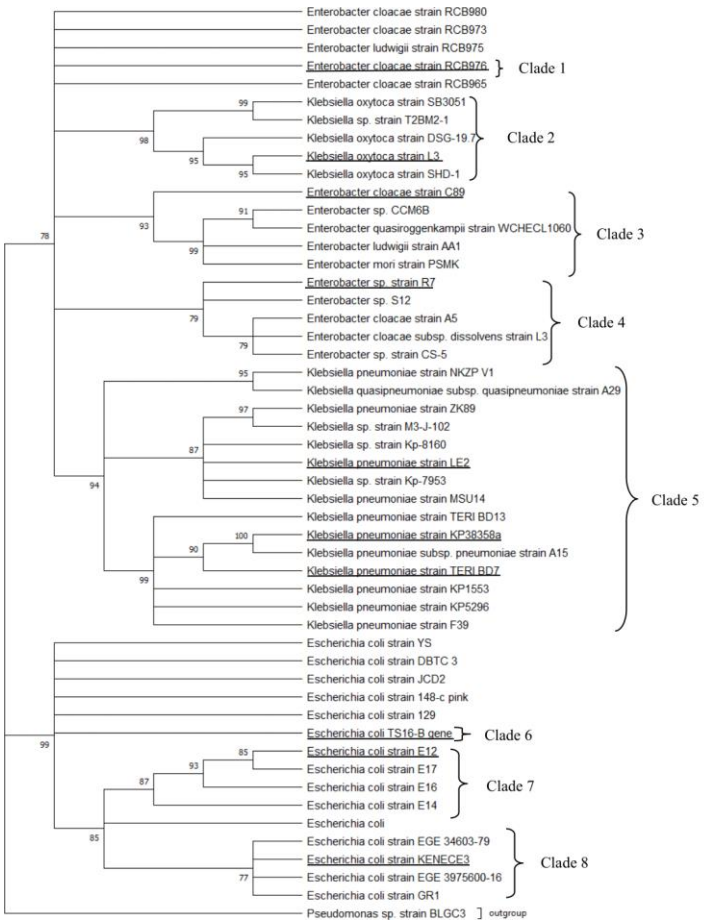

Figure 3 A phylogram portraying an evolutionary relatedness of pan-drug resistant Enterobacteriaceae reported in the present study with other reported Enterobacteriaceae available in Genbank by using 16s rRNA.

Table 8. Similarity identities and reported antimicrobial resistance information of isolated of the present study

AC	Species	Identities (%)	Reported AMR associated with the same strain	
KT261188.1	Enterobacter cloacae strain RCB976	95	Not reported	
OM236668.E1	nterobacter cloacae strain C89	96	Not reported	
KT261188.1	Enterobacter cloacae strain RCB976	96	Not reported	
MZ407768.E1	nterobacter sp. strain R7	99	ampicillin, amoxicillin-clavulanic acid, cefazolin, and cefoxitin,	[24]
MZ407768.E1	nterobacter sp. strain R7	97	ampicillin, amoxicillin-clavulanic acid, cefazolin, and cefoxitin,	[24]
MW418198K.1	lebsiella oxytoca strain L3	93	Not reported	
KM503148.K	lebsiella pneumoniae strain TERI BD7	96	Not reported	
KY040015.1	Klebsiella pneumoniae strain LE2	97	Not reported	
MK386773.K	lebsiella pneumoniae strain KP38358a	96	Not reported	
MK606097.E1	scherichia coli strain KENECE3	94	amoxicillin-clavulanic, ciprofloxacin, ofloxacin, gentamicin, meropenem, tetracycline	[25]
KY780347.1	Escherichia coli strain E12	97	Amoxicillin, Trimethoprim, fluoroquinolones tetracyclines	[26], [27]
LC487866.1	Escherichia coli TS16-B	98	gentamicin, tetracyclines, kanamycin, cefotaxime, ceftazidime, cefpirome, cefepime	[28]

DISCUSSION

Antimicrobial resistance pathogens are of global health concern, and this global health challenge is exacerbated by presence of multiple routes of transmission among people. One of the neglected hotspots is public transport, particularly in poor communities where there is overcrowding of people and poor hygiene and sanitation practices in public transport and may be a potential reservoir of antimicrobial-resistant of clinically importance [10]. Antimicrobial resistant microbes have been isolated in public transport in various places around the world [11], [12], [29]; however, limited information on AMR in public transport in Tanzania largest city of Dar es Salaam was lacking. Because previous reports were from cities with similar sanitation practices and population dynamics, it was hypothesized that AMR pathogens can be recovered from public transport in Dar es Salaam. As expected, the results are alarming because high abundance and antibiotic resistance in clinically relevant Enterobacteriaceae (E.coli, Klebsiella spp., and Enterobacter spp.) under the study were observed as shown in Table 4. Furthermore, the MDR isolates were common and very prevalent (Table 6).

ABUNDANCE OF ENTEROBACTERIACEAE OF CLINICAL IMPORTANCE

This is the first study to report presence of clinically relevant enterobacteriaceae in public transports of Dar es Salaam, Tanzania. A total of 131 isolates were recovered from just 100 pooled samples from ten different sampling points, which constitutes Klebsiella spp. (52%), E. coli (37%), and Enterobacter spp. (11%). Although the number may seem to be low, it should be taken into consideration that the study involved only clinically relevant Enterobacteriaceae, and therefore the presence of these diseases causing agent is of public health concern. Also, previous studies from Europe, Asia, America, and some African nations like Ghana and Ethiopia have shown the presence of Enterobacteriaceae in public transportation buses [9, 11, 12, 29].

The distribution of Enterobacteriaceae from this study, indicates that Klebsiella spp. and E. coli are the dominant species on public transport in Dar es Salaam in comparison to the abundance of Enterobacter spp (Table 3). High abundance of Klebsiella spp. and E.coli may be attributed to ecological niche preferences and abundace of the same species in the Dar es Salaam community. This explanation is corroborate by the same observation of high abundance of Klebsiella spp. (18%). and E. coli (10%) compared to Enterobacter spp. (7%) in a study that involved Automated Teller Machines Surfaces in Dar es Salaam, Tanzania [30]. Futhermore, similar observation on distribution of species of Enterobacteriaceae on public transport is consistent with previous reports highlighting the prominence of Klebsiella spp. and E. coli on other frequently hand-touched surfaces [30–33]. However, despite this fact, the correlation of abundance of these microbes in public transport and communities of Dar es Salaam remain to be established.

Interestingly, the results did not reveal influence of sampling location on the abundance of the isolates. This suggests that the distribution of Enterobacteriaceae on frequently touched surfaces of buses is consistent across different bus stops in Dar es Salaam. The lack of location-specific variations in Enterobacteriaceae abundance may be attributed to factors such as the mobility of passengers, and the nature of surfaces being frequently touched. Another explanation of this obeservation may be attributed to the prevalence of ubiquitous strains that can be easily dispersed and survive in different environments, as well as their ability to form biofilms, which enhance their persistence on surfaces [34].

ANTIMICROBIAL RESISTANCE PROFILE OF ENTEROBACTERIACEAE OF CLINICAL RELEVANCE

The antibiotic susceptibility testing results indicated high level of resistance among the isolated Enterobacteriaceae. Generally, Enterobacteriaceae were highly resistant to Ampicillin and Amoxicillin, with 100% and 97% resistance, respectively as shown in Figure 3. This alarming antimicrobial resistance prevalence observed in this study is not surprising because similar results has been observed in previous studies [12, 35, 36]. Specifically, in a study

that was conducted in Ethiopia in public transport, 100% of E. coli and Enterobacter spp. were resistant to ampicillin [12]. The high antimicrobial resistance is primarily caused by the widespread use and misuse of these antibiotics, which creates selective pressure favoring the survival and spread of resistant strains, ultimately leading to the development of antimicrobial resistance [37]. This finding suggests that these two antibiotics (Ampicillin and Amoxicillin) may no longer be effective in treating infections caused by Enterobacteriaceae, highlighting the urgent need for alternative treatment options.

On the other hand, Enterobacteriaceae were relatively less resistant to Norfloxacin, Azithromycin, and Ceftriaxone where resistance to these antibiotics was 24%, 22%, and 21%, respectively (Figure 3). This low resistance observed against Norfloxacin, Azithromycin, and Ceftriaxone is consistent with previous reports [18], [35]. For example, in the study done in Ghana [35], it was found 20% of E. coli from the samples of human hands were resistant to Azithromycin and Ceftriaxone. Similarly, in a study that was conducted in Romania [36], the percentage of AMR among Enterobacteiaceae to cetriaxone was 25%. Also, the observation may be explained by differences in their mechanism of action. For instance, norfloxacin and azithromycin mechanism of action involve alterations in bacterial DNA gyrase/topoisomerase and ribosomal target sites respectively, unlike the beta-lactamase-mediated resistance mechanism of ampicillin [38]. However, it is important to note that even though these antibiotics are currently effective against a significant proportion of Enterobacteriaceae, continuous monitoring of resistance patterns is essential to identify any emerging trends and to guide treatment decisions.

In contrast to common observation from variouus reports on variability in restance pattern among the members of Enterobacteriaceae, in the present study findings did not demonstrate variabilty ($P>0.05$) in resistance profile among E. Coli, Klebsiella spp, and Enterobacter spp (Table 4). Normally, in other reports, higher AMR is observed in Klebsiella spp compared to E.coli and Enterobacter spp in most of antibiotics [39–42]. The possible explantion of the observation may include the Geographic or temporal variation; there could be regional or temporal differences in resistance patterns among Enterobacteriaceae. The present study might have focused on a specific geographic area or a particular time period where the resistance patterns differed from the reports cited. Antimicrobial resistance can vary based on local prescribing practices, antimicrobial usage, infection control measures, and other factors. Also, another possible explanation may be evolution of resistance; the resistance profiles of bacteria can change over time due to the emergence and spread of new resistance mechanisms. It is possible that the resistance patterns reported in the previous studies were based on data collected at an earlier time point, while the present study captured a different stage of resistance evolution. The constant adaptation of bacteria to antimicrobial agents can lead to changes in resistance patterns over time. These findings implies that efforts to combat antimicrobial resistance should target all members of this bacterial family, rather than focusing on specific species.

The study also investigated the influence of sampling location on the antimicrobial resistance profiles of Enterobacteriaceae. The results did not reveal a significant impact of sam-

pling location on the resistance profiles against the tested antibiotics. This suggests that the abundance and resistance patterns of Enterobacteriaceae were consistent across the different sampling locations. This observation may indicate the widespread distribution and dissemination of Enterobacteriaceae strains with similar resistance characteristics, regardless of the geographic location. However, interesting observations were made regarding specific sampling locations. For instance, all isolates from Tegeta bus station were susceptible to Norfloxacin, while isolates from Kigamboni bus station showed susceptibility to Ceftriaxone (Table 5). These findings suggest that certain locations may have variations in the antimicrobial resistance profiles of Enterobacteriaceae, potentially influenced by local factors such as antibiotic usage patterns or hygiene practices. For instance, it is possible that Norfloxacin and Ceftriaxone are less frequently used or prescribed in these specific areas, leading to a lower selective pressure for resistance development. It is worth noting that the observed susceptibility patterns of Enterobacteriaceae to Norfloxacin and Ceftriaxone at Tegeta bus station and Kigamboni bus station, respectively, might have important implications for empirical antibiotic therapy in these areas. These antibiotics could be considered as effective treatment options for Enterobacteriaceae infections in these specific locations, considering their high susceptibility level.

Furthermore, the study assessed the prevalence of MDR isolates among the Enterobacteriaceae. The results indicated a high prevalence of MDR strains, with 98% of the isolates classified as MDR, which is similar to a study done in Ethiopia, reported as 92.1% [43], and in Gondar, reported as (87.4%) [44]. Among them, 87% were extensively drug-resistant (XDR), and 12% were pandrug-resistant (PDR). This might be attributed to various factors, one possible explanation is the misuse and overuse of antibiotics, which promotes the development and spread of drug-resistant strains. Inadequate infection control practices and poor sanitation in healthcare settings can also contribute to the dissemination of MDR Enterobacteriaceae. These findings highlight the urgent need for comprehensive antimicrobial stewardship programs and infection control strategies to prevent the further dissemination of MDR Enterobacteriaceae in the public transport environment. These findings align with the global trend of increasing antimicrobial resistance of Enterobacteriaceae in various healthcare and community settings that have reported the high prevalence of MDR, posing a significant threat to public health[43], [44]. The emergence and spread of MDR strains pose significant challenges to the treatment of infectious diseases, as they limit the effectiveness of available antibiotics. Therefore, strategies such as improved hygiene practices, appropriate antibiotic use, and public health interventions are crucial to address this growing public health threat. Moreover, a significant proportion of the MDR isolates in the present study were extensively drug-resistant (XDR) and pandrug-resistant (PDR), highlighting the urgent need for effective infection control strategies and the development of new antimicrobial agents.

GENOTYPIC AND PHENOTYPIC AGREEMENT AMONG ENTEROBACTERIACEAE

To understand the underlying mechanisms of resistance, genotypic analysis was performed to detect commonly reported antibiotic resistance genes. The results revealed a strong positive correlation between phenotypic resistance and genotypic resistance (r_s = 0.66, P<0.05), indicating that the presence of resistance genes in the isolates corresponds well with their observed resistance phenotype. The agreement between genotypic and phenotypic resistance provides valuable insights into the genetic basis of antibiotic resistance in Enterobacteriaceae. This suggests that genotypic screening can be a reliable indicator of phenotypic resistance in Enterobacteriaceae isolates. In comparison to other studies, these finding are consistent with[22], [1,45–47]. For instance, the study that was done in Cameroon[46], the resistance gene carriage by Enterobacteriaceae isolated from broilers correlated significantly to reduced susceptibility for quinolone and aminoglycoside resistance.

However, it is noteworthy that in some Enterobacteriaceae isolates, resistance genes were absent despite demonstrating phenotypic resistance to specific antibiotics (Table 7). For example, one interesting finding is the lack of concordance between the Gentamicin and Aac(3)-IV resistance gene among all isolates, as they exhibited 0% agreement. This suggests that phenotypic resistance to Gentamicin may be attributed to mechanisms other than the presence of the Aac(3)-IV resistance gene. This finding is fairly similar to study in Iran[19], in which Aac(3)-IV resistance gene was not detected from E. coli isolated from slaughtered commercial chickens. In contrast, the Trimethoprim/Sulfamethoxazole resistance gene, dfrA1, displayed 100% concordance between phenotypic and genotypic resistance. This indicates that the presence of the dfrA1 gene is strongly associated with the observed phenotypic resistance to Trimethoprim/Sulfamethoxazole in all isolates (Table 7). These discrepancies between genotypic and phenotypic resistance could be attributed to various factors, including the presence of alternative resistance mechanisms that were not evaluated in this study. It is possible that other resistance genes or mechanisms, not targeted in the PCR assay, were responsible for the observed phenotypic resistance in these isolates. Additionally, epigenetic modifications, such as gene regulation or expression, may also contribute to the observed differences between genotypic and phenotypic resistance

CONCLUSION

The results of the present study indicate the widespread contamination of public transport surfaces with clinically important Enterobacteriaceae, posing a potential threat to public health. Specifically, this study sheds light on the abundance and antimicrobial susceptibility of Enterobacteriaceae in the public transport system of Dar es Salaam, Tanzania. The high prevalence of Enterobacteriaceae, coupled with the alarming rates of antibiotic resistance, highlights the need for comprehensive surveillance, improved hygiene practices, and effective antimicrobial stewardship in public transport settings. These findings serve as a

valuable baseline for future studies and emphasize the importance of addressing the growing threat of antimicrobial resistance in both healthcare and community settings.

Conflict of interest statement

The authors declare that there is no conflict of interest

Funding:

The study did not receive any external fund

Authors' contributions

All authors have contributed to concept and design of the study. GH analyzed and interpreted all data generated in the study. All authors read and approved the final manuscript.

Availability of data and materials

In this study, all the data and materials are included within this article. If you need raw data you should contact the authors.

ACKNOWLEDGEMENTS

We would like to appreciate the University of Dar es Salaam, particularly the Department of Molecular Biology and Biotechnology (MBB) to allow this work to be conducted in their Laboratory under guidance of MBB technical staff.

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