3 Biotech (2020) 10:238
https://doi.org/10.1007/s13205-020-02219-z

ORIGINAL ARTICLE

Unravelling the antibiotic and heavy metal resistome of a chronically polluted soil

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Received: 27 February 2020 / Accepted: 24 April 2020 / Published online: 7 May 2020

Abstract

The antibiotic and heavy metal resistome of a chronically polluted soil (3S) obtained from an automobile workshop in Ilorin, Kwara State, Nigeria was deciphered via functional annotation of putative ORFs (open reading frames). Functional annotation of antibiotic and heavy metal resistance genes in 3S metagenome was conducted using the Comprehensive Antibiotic Resistance Database (CARD), Antibiotic Resistance Gene-annotation (ARG-ANNOT) and Antibacterial Biocide and Metal Resistance Gene Database (BacMet). Annotation revealed detection of resistance genes for 15 antibiotic classes with the preponderance of beta lactamases, mobilized colistin resistance determinant (mcr), glycopepetide and tetracycline resistance genes, the OqxBgb and OqxA RND-type multidrug efflux pumps, among others. The dominance of resistance genes for antibiotics effective against members of the *Enterobacteriaceae* indicate possible contamination with faecal materials. Annotation of heavy metal resistance genes revealed diverse resistance genes responsible for the uptake, transport, detoxification, efflux and regulation of copper, zinc, cadmium, nickel, chromium, cobalt, mercury, arsenic, iron, molybdenum and several others. Majority of the antibiotic and heavy metal resistance genes detected in this study are borne on mobile genetic elements, which facilitate their spread and dissemination in the polluted soil. The presence of the heavy metal resistance genes is strongly believed to play a major role in the proliferation of antibiotic resistance genes. This study has established that soil is a huge repertoire of antibiotic and heavy metal resistome and due to the intricate link between human, animals and the soil environment, it may be a major contributor to the proliferation of multidrug-resistant clinical pathogens.

Keywords Heavy metals · Antibiotics · Shotgun metagenomics · Heavy metal resistome · Antibiotic resistome

Introduction

Soil, the naturally occurring unconsolidated mineral and organic material is home to a huge and diverse population of microorganisms due to its tremendous range of habitats determined by a complex interplay between several biotic and abiotic factors. Extensive inundation of the soil environment with a coterie of anthropogenic pollutants such as heavy metals and antibiotics imposes selective pressure on the microbial community and forces it to develop counteractive strategies for adaptation, survival and successful reproduction (Chandra and Kumar 2017). While intrinsic resistance against natural antibiotics produced by soil microorganisms could select for microorganisms with antibiotic resistance phenotypes, acquired resistance caused by the presence in the soil of potentially offensive compounds and conditions might select for specific and non-specific mechanisms of resistance (Martin and Liras 1989; Hopwood 2007; Tahlan et al. 2007; Allen et al. 2010).

It is widely believed that poor sanitary conditions and urban infrastructure, lack of regulatory and enforcement policies, and corruption, particularly in developing countries have allowed unrestricted environmental release of antibiotics from diverse point and non-point sources (Collignon et al. 2015). This has escalated the incidences of multidrug resistance particularly among clinical isolates due to the intricate link that exist between humans, animals and their environment (Allen et al. 2010). In soil, acquired resistance by microorganisms may be traced to various human

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13205-020-02219-z) contains supplementary material, which is available to authorized users.

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activities such as excretion of antibiotics via open defecation and urination by medicated humans and pets; running urban sewage carrying antibiotics from houses and hospitals as well as wastewaters from fish ponds in open, exposed channel, and sometimes directly onto the streets (Amabile-Cuevas 2016). Others include leachates from antibiotic-containing wastes; surface runoff from solid waste municipal dumpsite; and the usage of antibiotic-containing wastewater and livestock manure in agriculture (Arun et al. 2017).

Microorganisms have deployed various strategies to counteract the toxic effects of antibiotics. These include active efflux of the antibiotic from the microbial cell; modification of antibiotic targets; enzymatic modification of the antibiotic; enzymatic degradation of the antibiotic; creating bypass or alternative metabolic pathways to those inhibited by the antibiotic; overproduction of the target enzyme; and access restriction of antibiotic to target sites via permeability changes in the bacterial cell wall (van Hoek et al. 2011; Panesyan et al. 2015).

Naturally, soil can have high concentrations of heavy metals due to weathering of parental material resulting in high concentrations of heavy metal minerals (Kamal et al. 2010). However, anthropogenic activities such as mining, long term application of sewage sludge, fertilizers, pesticides and herbicides, composted municipal solid wastes, improper waste disposal practices, smelting, wastewater irrigation, manufacturing and agrochemicals, and indiscriminate disposal of spent oils rich in heavy metals have exacerbated the heavy metals burden in the soil (Sandaa et al. 1999; Gans et al. 2005; Khan et al. 2017). Microorganisms have developed efficient detoxification strategies to counteract the toxic effects of heavy metal stress. These include intracellular sequestration, export, reduced permeability, extracellular sequestration, and extracellular detoxification (Rough et al. 2005). The resistance determinants are mostly encoded on mobile genetic elements such as plasmid and transposons, which facilitate promiscuous transmission and dissemination of the genes among members of the microbial community via horizontal gene transfer (Silver and Walderhaug 1992; Osborn et al. 1997).

Evidences abound suggesting that co-selection mechanisms such as co-resistance, cross-resistance and co-regulation contributed significantly to the maintenance and promotion of antibiotic resistance in the microbial population in the absence of antibiotics (Pal et al. 2015a, b, 2017). Cross-resistance mechanism, which occurs when a single mechanism provides resistance to different compounds, have been reported for efflux pumps with broad substrate specificity, which protect the cell from both antibiotics and heavy metals (Mata et al. 2000; Nies 2003a, b; Blanco et al. 2016). Co-selection of antibiotic and heavy metal resistance is also promoted by the co-resistance mechanism, which occurs when two or more different resistance genes are co-located on a plasmid or a transposon. This mechanism has been fingered in co-resistance to copper, erythromycin, vancomycin, and tetracycline (Hasman and Aarestrup 2002; Amachawadi 2011); copper, silver, β-lactam and fluoroquinolone (Fang et al. 2016); cadmium, zinc, and methicillin (Cavaco et al. 2011); copper, silver, mercury, colistin, ampicillin, sulphonamide, tetracycline, streptomycin, and chloramphenicol (Campos et al. 2016), and several others. Furthermore, there are reports that microorganisms use regulatory proteins, which control the expression of heavy metal resistance genes to confer resistance to antibiotics, a phenomenon termed co-regulatory mechanism (Perron et al. 2004; Pal et al. 2017). The concept of co-selection has heightened the rate of spread and dissemination of antibiotic resistance genes in the environments, which, in turn has increase the emergence of multidrug resistance clinical pathogens, constituting an alarming threat to public and environmental health.

The use of shotgun metagenomics to explore perturbed and pristine soil environments has gained ascendancy due to its ability to reveal intricate details about the structural and functional properties of the studied environment. In this study, an attempt was made to use shotgun metagenomics to unravel the antibiotic and heavy metal resistome of a chronically polluted soil and highlight the implications of their presence on public and environmental health.

Materials and methods

Sampling site description, microcosm set up, and determination of residual hydrocarbons

Hydrocarbon-polluted soil samples were collected from an automobile workshop at Taiwo, Ilorin, Nigeria. The coordinates of the sampling site were latitude 8°28′ 42.4″ N and longitude 4°32′15.6″ E. The site has a long history of hydrocarbon contamination spanning a period of more than 10 years. Prior to this time, small-scale agriculture is the predominant activity in this area. Until date, because the workshop is situated on an open expanse of land, traditional cattle rearing, as well as poor hygiene practices such as open defecation and urination is very common in this area. Samples were collected at a depth of 10–12 cm with a sterile hand trowel, sieved (4 mm) and thoroughly mixed in a large plastic bag to avoid variability among the results of replicate soil samples. Details on the soil microcosm (polluted soil, 3S) set up, incubation conditions, and residual hydrocarbons have been reported previously (Salam and Ishaq 2019). The physicochemical properties of the polluted soil indicate a pH of 6.76, organic matter content of 1.38%, total nitrogen of 0.13%, and phosphorus and potassium content of 6.38 and 0.15 mg/kg, respectively (Salam and Ishaq 2019).

DNA extraction, library construction, sequencing, and metagenome properties

Total DNA used for metagenomic analysis was extracted directly from 3S soil microcosm (0.25 g) using ZYMO soil DNA extraction Kit (Model D 6001, Zymo Research, USA) following the manufacturer's instructions. DNA concentration and quality were ascertained using NanoDrop spectrophotometer and electrophoresed on a 0.9% (w/v) agarose gel, respectively. Shotgun metagenomic of 3S soil microcosm was prepared using the Illumina Nextera XT sample processing kit and sequenced on a MiSeq. The protocols for total DNA preparation for Illumina shotgun sequencing were as described previously (Salam 2018; Salam and Ishaq 2019). The sequence reads of 3S metagenome were deposited on the MG-RAST server with the ID 4704694.3 and can be accessed with the link https://www.mg-rast.org/linkin.cgi?project=mgp18598.

Sequence reads from the 3S microcosm set up were assembled individually by VelvetOptimiser v2.2.5, and the resulting contigs fed into the MG-RAST metagenomic analysis pipeline. The sequences were assembled into 1239 unique contigs with a total of 314,848 bp, an average sequence length of 254 ± 66 bp, and the mean GC content of 61 ± 6%, respectively. After dereplication and quality control by the MG-RAST, sequence reads in 3S metagenome reduced to 1,064 with 260,627 bp, and an average sequence length of 245 ± 55 bp (Salam and Ishaq 2019).

Accession number

The data, metadata and sequence reads of the metagenome used in this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB36986 (https://www.ebi.ac.uk/ena/data/view/PRJEB36986).

Functional analyses of 3S metagenome for antibiotic and heavy metal resistome

Gene calling was performed on the 3S contigs using FragGeneScan (Rho et al. 2010) to predict open reading frames (ORFs). The ORFs were functionally annotated for antibiotic and heavy metal resistance genes using the Comprehensive Antibiotic Resistance Database (CARD, McArthur et al. 2013), the Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT V6; Gupta et al. 2014), and BacMet (Pal et al. 2014), a function-specific bioinformatics resource for the detection of antibacterial biocide and metal-resistance genes. In CARD, the Resistance Gene Identifier (RGI 5.1.0, CARD 3.0.7) was used to predict antibiotic resistome from the protein sequences (ORFs) of the 3S metagenome. Strict significance was calculated based on CARD curated bitscore cut-offs for metagenomic reads, which allow prediction of partial genes. In ARG-ANNOT, 3S protein sequences (ORFs) were fed into the functional annotation resource, which uses NCBI blastp program to annotate the protein sequences and provide information on the existing and putative new antibiotic resistance genes (ARG) detected in the protein sequences based on coverage and similarity. In BacMet, the protein sequences (ORFs) were presented as a query to the BacMet database (version 2.0) using default parameters for identification of metal-resistance genes. A modified stand-alone version of the BLAST program (NCBI, version 2.2.2) implemented in the BacMet webserver was used for similarity searches against the BacMet sequence databases.

Results

Functional annotation of 3S metagenome ORFs using CARD, ARG-ANNOT and BacMet revealed the presence of antibiotic resistance genes for several antibiotic classes and resistance genes responsible for transport, efflux, and detoxification of heavy metals.

Antibiotic resistance genes (ARG)

β-lactamase genes

In 3S metagenome, 144 β-lactamase genes cutting across the four Ambler classes (A-D) were recovered (Fig. 1). In class A, 60 genes were detected. The predominant genes include *blaCTX-M* (146; 15.7%), *blaTEM* (142; 15.2%); *blaSHV* (126; 13.5%); *blaGES* (62; 6.7%), and *blaVEB* (59; 6.3%). Forty-six (46) β-lactamase genes belonging to class B were retrieved from 3S metagenome with the preponderance of *blaNDM* (151; 22.1%), *blaVIM* (106; 15.5%); *blaGOB* (55; 8.1%); *blaLRA* (46; 6.7%); and *blaB* (44; 6.5%), respectively. Twenty-eight (28) genes belonging to class C were recovered from 3S metagenome with *blaCMY* (273; 23.8%), *blaLEN* (161; 14.0%), *blaOKP* (135; 11.7%), *blaPDC* (110; 9.6%), and *blaADC* (74; 6.4%) preponderant. Only nine (9) genes belonging to class D were retrieved from 3S metagenome with the dominance of *blaOXA* gene ($n = 507$) constituting 91.7% of class D β-lactamase genes recovered (Fig. 1). An integral membrane β-lactamase regulatory protein *blaR1*, and 17 variants of penicillin-binding protein PBP were also recovered from the metagenome.

Aminoglycoside and MLS (macrolide-lincosamide-streptogramin) resistance genes

Fifty-six (56) aminoglycoside resistance genes were recovered from 3S metagenome (Fig. 2a). The recovered genes belong to the major classes of aminoglycoside resistance

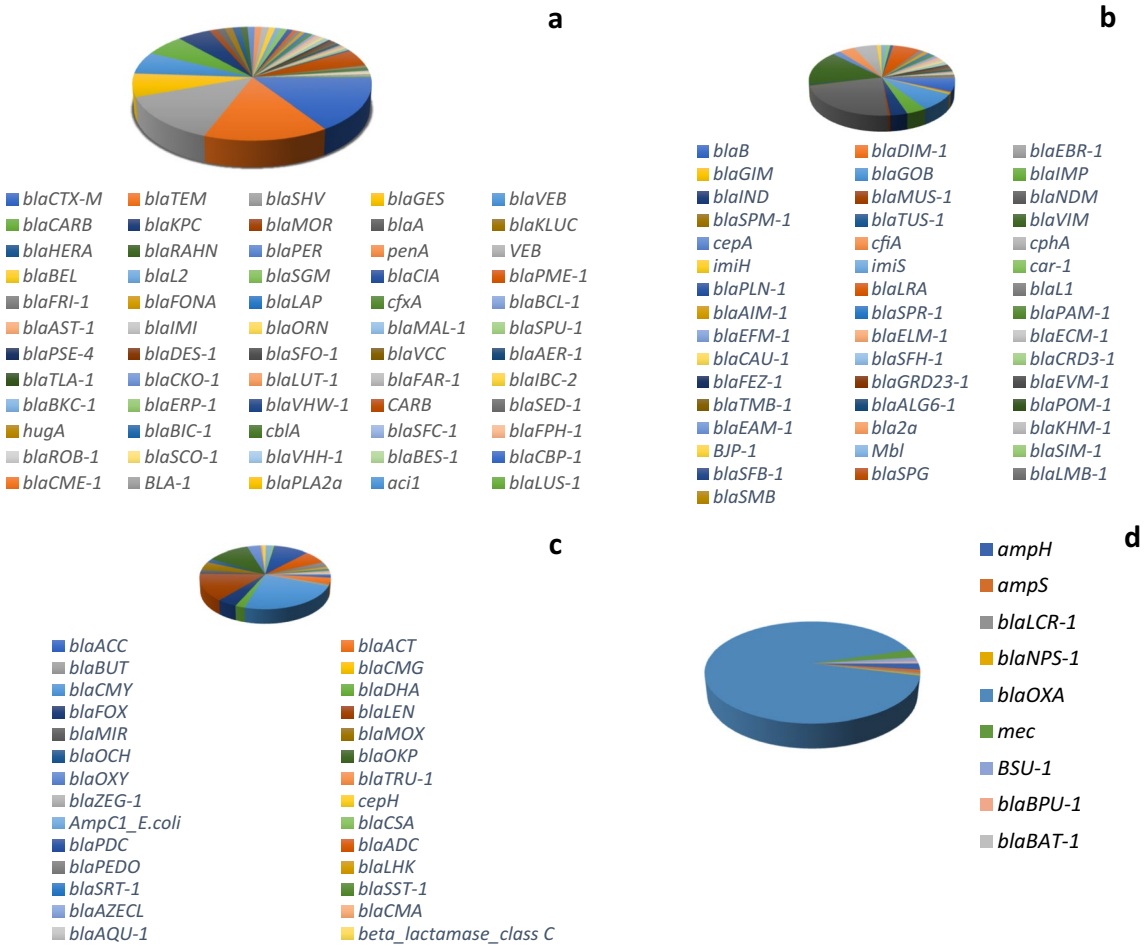

Fig. 1 Distribution of resistance genes encoding class A (**a**), class B (**b**), class C (**c**), and class D β-lactamases in 3S metagenome (**d**). In class A, *blaTEM, blaCTX-M*, and *blaSHV* are the dominant resistance genes. In class B, *blaNDM, blaVIM* and *blaGOB* are the resistance genes with the highest representation. Class C β-lactamases are dominated by *blaCMY, blaLEN, blaOKP* and *blaPDC* while in class D, *blaOXA* is predominant. Majority of class A β-lactamases and *blaOXA* detected in this study belong to extended spectrum β-lactamases (ESBLs)

genes. This include acetyltransferase ACT (*aac; sat; apmA*), nucleotidyltransferase/adenyltransferase NUT (*aad; sph; str; ant*), methyltransferase MET (*rmt; spc; armA*), and phosphotransferase (*aph; vph*). Distribution of aminoglycoside resistance genes in 3S metagenome revealed the preponderance of *aadA* (80; 16.6%), *aac*(*6′*) (58; 12.1%), *aac3* (48; 10.0%), *strB* (45; 9.4%), and *aph(3′)* (24; 5.0%), respectively.

Eighty-five (85) MLS resistance genes responsible for resistance mechanisms such as rRNA methylase, efflux and inactivating genes were retrieved from 3S metagenome (Fig. 2b). Of these, 37 genes (*cfr; erm; tlr; myr; emt*) is responsible for rRNA methylase, 23 genes (*Isa; lmr; mef; msr; ole; srm; vga; sal*(*A*)) is responsible for efflux, and 25 genes (esterase (*ere)*; lyase (*vgb*); transferase (*lnu, vat, lin*); phosphorylase (*mph*)) is responsible for inactivating genes. Distribution of MLS resistance genes in the metagenome revealed the predominance of *vga*(*A*) (23; 5.1%), *lin* (16; 3.6%), *ole*(*B*) (15; 3.3%), *vga*(*D*) (14; 3.1%), and *msr*(*E*) (13; 2.9%), respectively. Other MLS resistance gene detected only in CARD is *macB*, a macrolide export ATP-binding cassette (ABC) efflux pump (Table 1).

Colistin, phenicol, rifampin, and fosfomycin resistance genes

Sixty-one (61) plasmid-borne mobilized colistin resistance determinant (*mcr*) comprising of *mcr-1* to *mcr-9* and their variants were recovered in 3S metagenome (Fig. 3a). The distribution of the mcr variants in the metagenome in decreasing order (abundance value in parentheses) indicate *mcr-3* (27), *mcr-1* (18), *mcr-4* (6), *mcr-5* (3), *mcr-2* (2), *mcr-8* (2), *mcr-6* (1), *mcr-7* (1), and *mcr-9* (*1*), respectively.

Forty-nine (49) phenicol resistance genes comprising *cat* (28), *cml* (10), *cmr* (4), *cpt* (2), *dha1* (1), *fexA* (1), *floR* (1),

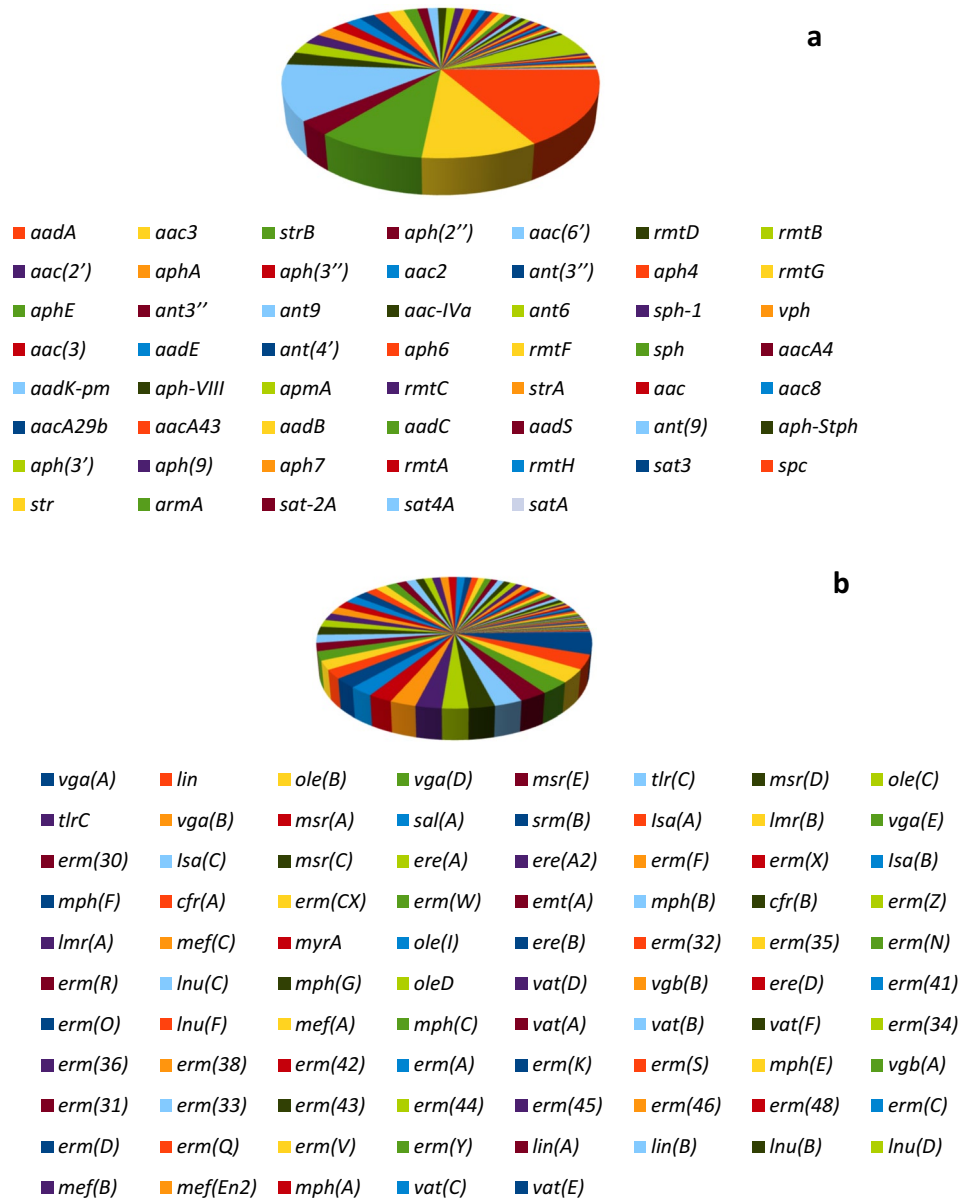

Fig. 2 Distribution of aminoglycoside (**a**) and MLS (**b**) resistance genes in 3S metagenome. The dominant aminoglycoside resistance genes are those belonging to the acetyltransferases ACT (*aac*) and the nucleotidyltransferase/adenyltransferase NUT *(aadA, strB*). Based on MLS resistance mechanisms, 37 genes (*cfr; erm; tlr; myr; emt*) is responsible for rRNA methylase, 23 genes (*lsa; lmr; mef; msr; ole; srm; vga; sal*(*A*)) is responsible for efflux, and 25 genes (esterase (*ere*); lyase (*vgb*); transferase (*lnu, vat, lin*); phosphorylase (*mph*)) is responsible for inactivating genes

and *pexA* (1) genes were recovered from 3S metagenome (Fig. 3b). Based on resistance mechanism, 31 genes (*cat, cmlV, cpt*) are responsible for phenicol inactivation, while the remaining 18 genes (c*mlA, cmlB, cmlR, cmr, cmrA, bcr1, dha1, fexA, floR, pexA*) are responsible for phenicol efflux.

Nine (9) rifampin resistance genes were retrieved from 3S metagenome (Fig. 3c). These include the rifampin-inactivating phosphotransferase (*rph, rphD*), rifampin resistance protein (*rif*), rifampin monooxygenase (*iri*), and integron (*arr2, arr4, arr5, arr7*) and plasmid (*arr3*) -encoded rifampin ADP ribosyltransferase. While the resistance mechanism of the 9 genes is antibiotic inactivation, annotation using CARD reveal the detection of *rpoB* gene (a β-subunit of RNA polymerase) (Table 1) whose mutations confers resistance to rifampin due to antibiotic target alteration and replacement.

Seventeen (17) fosfomycin resistance genes were retrieved from 3S metagenome (Fig. 3d). These include the fosfomycin modifying genes (*fosA, fosB, fosC, fosD, fosE,*

Table 1 Antibiotic resistome detected in 3S metagenome (using CARD) involved in resistance to different classes of antibiotics

ORF ID	ARO term	SNP	Detection criteria	AMR gene family	Drug class	Resistance mechanism	% identity of matching region	% length of reference sequence
NODE_119_length_161_cov_1.937888.1	arlR		Protein homolog model	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone antibiotic, acridine dye	Antibiotic efflux	44.9	34.25
NODE_206_length_233_cov_1.257511.1	macB		Protein homolog model	ATP-binding cassette (ABC) antibiotic efflux pump	Macrolide antibiotic	Antibiotic efflux	41.43	11.34
NODE_358_length_253_cov_1.557312.1	*Corynebacterium striatum* tetA		Protein homolog model	Major facilitator superfamily (MFS) antibiotic efflux pump	Penam, tetracycline antibiotic	Antibiotic efflux	61.22	20.47
NODE_453_length_289_cov_1.944637.1	macB		Protein homolog model	ATP-binding cassette (ABC) antibiotic efflux pump	Macrolide antibiotic	Antibiotic efflux	34.55	18.17
NODE_495_length_248_cov_1.419355.1	TaeA		Protein homolog model	ATP-binding cassette (ABC) antibiotic efflux pump	Pleuromutilin antibiotic	Antibiotic efflux	48.68	16.05
NODE_581_length_262_cov_1.881679.1	MuxB		Protein homolog model	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, monobactam, tetracycline antibiotic, aminocoumarin antibiotic	Antibiotic efflux	59.8	10.45
NODE_694_length_211_cov_1.848341.1	*Bifidobacterium adolescentis* rpoB mutants conferring resistance to rifampicin		Protein homolog model	Rifamycin-resistant beta-subunit of RNA polymerase (rpoB)	Rifamycin antibiotic	Antibiotic target alteration, antibiotic target replacement	57.3	7.67
NODE_844_length_262_cov_2.870229.1	MuxB		Protein homolog model	resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, monobactam, tetracycline antibiotic, aminocoumarin antibiotic	antibiotic efflux	64.08	10.45
NODE_893_length_142_cov_1.915493.1	macB		Protein homolog model	ATP-binding cassette (ABC) antibiotic efflux pump	macrolide antibiotic	Antibiotic efflux	38.78	10.71
NODE_1047_length_172_cov_1.453488.1	msbA		Protein homolog model	ATP-binding cassette (ABC) antibiotic efflux pump	Nitroimidazole antibiotic	Antibiotic efflux	32.73	13.4
NODE_1068_length_352_cov_1.073864.1	vanSB		Protein homolog model	vanS, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration	32.41	31.1
NODE_1294_length_172_cov_1.883721.1	TRU-1		Protein homolog model	TRU beta-lactamase	Cephalosporin, penam	Antibiotic inactivation	36.84	20.68

Table 1 (continued)

ORF ID	ARO term	SNP	Detection criteria	AMR gene family	Drug class	Resistance mechanism	% identity of matching region	% length of reference sequence
NODE_1445_length_151_cov_2.655629.1	golS		Protein homolog model	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Monobactam, carbapenem, cephalosporin, cephamycin, penam, phenicol antibiotic, penem	Antibiotic efflux	31.34	46.75
NODE_1538_length_228_cov_4.052631.1	evgS		Protein homolog model	Major facilitator superfamily (MFS) antibiotic efflux pump, resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic, penam, tetracycline antibiotic	Antibiotic efflux	35.14	8.1
NODE_1553_length_166_cov_2.765060.1	mdtA		Protein homolog model	resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminocoumarin antibiotic	Antibiotic efflux	50	18.55
NODE_1650_length_140_cov_10.214286.1	vanE		Protein homolog model	Glycopeptide resistance gene cluster, van ligase	Glycopeptide antibiotic	Antibiotic target alteration	40	19.03
NODE_1661_length_323_cov_3.764706.1	vanRF		Protein homolog model	Glycopeptide resistance gene cluster, vanr	Glycopeptide antibiotic	Antibiotic target alteration	28.95	55.84
NODE_1668_length_361_cov_1.047091.1	*Bifidobacterium adolescentis* rpoB mutants conferring resistance to rifampicin		Protein homolog model	Rifamycin-resistant beta-subunit of RNA polymerase (rpoB)	Rifamycin antibiotic	Antibiotic target alteration, antibiotic target replacement	47.66	11.89
NODE_1676_length_210_cov_2.000000.1	rpoB2		Protein homolog model	Rifamycin-resistant beta-subunit of RNA polymerase (rpoB)	Rifamycin antibiotic	Antibiotic target alteration, antibiotic target replacement	66.29	7.83
NODE_2311_length_373_cov_1.018767.1	vanSB		Protein homolog model	vanS, glycopeptide resistance gene cluster	Glycopeptide antibiotic	Antibiotic target alteration	27.34	32.66
NODE_2482_length_334_cov_1.107784.1	sul2		Protein homolog model	Sulfonamide resistant sul	Sulfonamide antibiotic	Antibiotic target replacement	60.87	10.33
NODE_2884_length_294_cov_1.132653.1	tetB(60)		Protein homolog model	ATP-binding cassette (ABC) antibiotic efflux pump	Tetracycline antibiotic	Antibiotic efflux	36	19.69

Table 1 (continued)

ORF ID	ARO term	SNP	Detection criteria	AMR gene family	Drug class	Resistance mechanism	% identity of matching region	% length of reference sequence
NODE_2918_length_262_cov_4.240458.1	mdtC		Protein homolog model	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminocoumarin antibiotic	ANTIBIOTIC efflux	76.47	10.63
NODE_3078_length_211_cov_3.127962.1	*Bifidobacterium adolescentis* rpoB mutants conferring resistance to rifampicin		Protein homolog model	Rifamycin-resistant beta-subunit of RNA polymerase (rpoB)	Rifamycin antibiotic	Antibiotic target alteration, antibiotic target replacement	56.82	7.67
NODE_3092_length_219_cov_2.251142.1	hmrM		Protein homolog model	Multidrug and toxic compound extrusion (MATE) transporter	Fluoroquinolone antibiotic, acridine dye	Antibiotic efflux	28.57	20.47
NODE_3146_length_148_cov_1.020270.1	smeS		Protein homolog model	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminoglycoside antibiotic, cephalosporin, cephamycin, penam	Antibiotic efflux	43.48	15.2
NODE_3209_length_193_cov_1.471503.1	macB		Protein homolog model	ATP-binding cassette (ABC) antibiotic efflux pump	Macrolide antibiotic	Antibiotic efflux	29.41	13.35
NODE_3429_length_199_cov_1.839196.1	S*treptomyces rishiriensis* parY mutant conferring resistance to aminocoumarin		Protein homolog model	Aminocoumarin resistant parY	Aminocoumarin antibiotic	Antibiotic target alteration	44.71	12.52
NODE_3434_length_241_cov_1.609959.1	otr(A)		Protein homolog model	Tetracycline-resistant ribosomal protection protein	Tetracycline antibiotic	Antibiotic target protection	35.87	15.38
NODE_3458_length_256_cov_1.250000.1	macB		Protein homolog model	ATP-binding cassette (ABC) antibiotic efflux pump	Macrolide antibiotic	Antibiotic efflux	32.91	16.61
NODE_3466_length_199_cov_1.934673.1	*Streptomyces rishiriensis* parY mutant conferring resistance to aminocoumarin		Protein homolog model	Aminocoumarin resistant parY	Aminocoumarin antibiotic	Antibiotic target alteration	47.73	12.52
NODE_2500_length_249_cov_1.602410.1	*Escherichia coli* fabI mutations conferring resistance to isoniazid and triclosan	F203L	Protein variant model	Antibiotic resistant fabI	Isoniazid, triclosan	Antibiotic target alteration	30.77	37.4

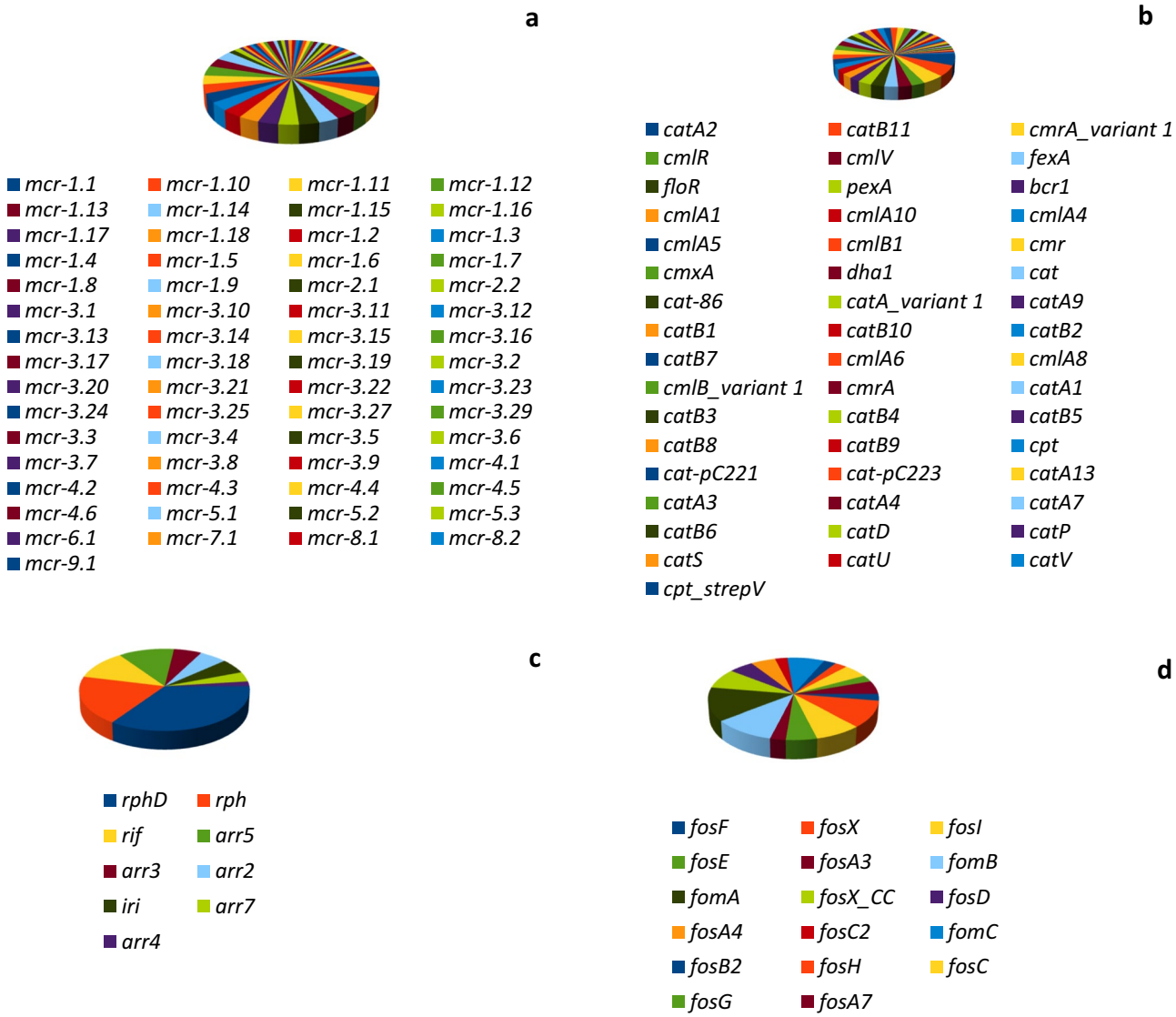

Fig. 3 Distribution of colistin (**a**), phenicol (**b**), rifampin (**c**), and fosfomycin (**d**) resistance genes in 3S metagenome. Colistin resistance genes are dominated by the mobilized colistin resistance (mcr) determinants, mcr-1 and mcr-3. Based on phenicol resistance mechanisms, 31 genes (*cat, cmlV, cpt*) are responsible for phenicol inactivation, while 18 genes (*cmlA, cmlB, cmlR, cmr, cmrA, bcr1, dha1, fexA, floR, pexA*) are responsible for phenicol efflux. Rifampin-inactivating phosphotransferase (rph, rphD) dominated rifampin resistance genes in 3S, however, all the resistance genes inactivate rifampin antibiotic. Fosfomycin resistance genes include fosfomycin modifying genes (*fosA, fosB, fosC, fosD, fosE, fosF, fosG, fosH, fosX*) and the fosfomycin kinases (*fomA, fomB, fomC*)

fosF, fosG, fosH, fosX) and the fosfomycin kinases (*fomA, fomB, fomC*). The resistance mechanism of these genes is believed to be antibiotic inactivation.

Tetracycline, glycopeptide, fluoroquinolone, and oxazolidinone resistance genes

Fifty-four (54) tetracycline resistance genes were recovered from 3S metagenome (Fig. 4a). These are classified on the basis of resistance mechanism into resistance genes for ribosomal protection proteins (*otrA, tetM, tetO, tetS, tetT, tetW, tet(32), tet(36), tet(44), tetQ*), resistance genes for tetracycline-inactivating enzymes (*tet(34), tet(37), tet(47), tet(48), tet(49), tet(50), tet(51), tet(52), tet(53), tet(54), tetX*), and resistance genes for tetracycline efflux (*otrB, otrC, tetA, tetB, tetAB, tetC, tetD, tetE, tetG, tetH, tetJ, tetL, tetR, tcr-3, tet(30), tet(31), tet(33), tet(35), tet(38), tet(39), tet(40), tet(41), tet(42), tet(43), tet(45), tet(57), tet(58), tet(59), tet(60), tetU, tetV, tetY, tetZ*).

Eighty-one glycopeptide resistance genes were retrieved from 3S metagenome (Fig. 4b). These are classified into resistance genes responsible for the production of modified

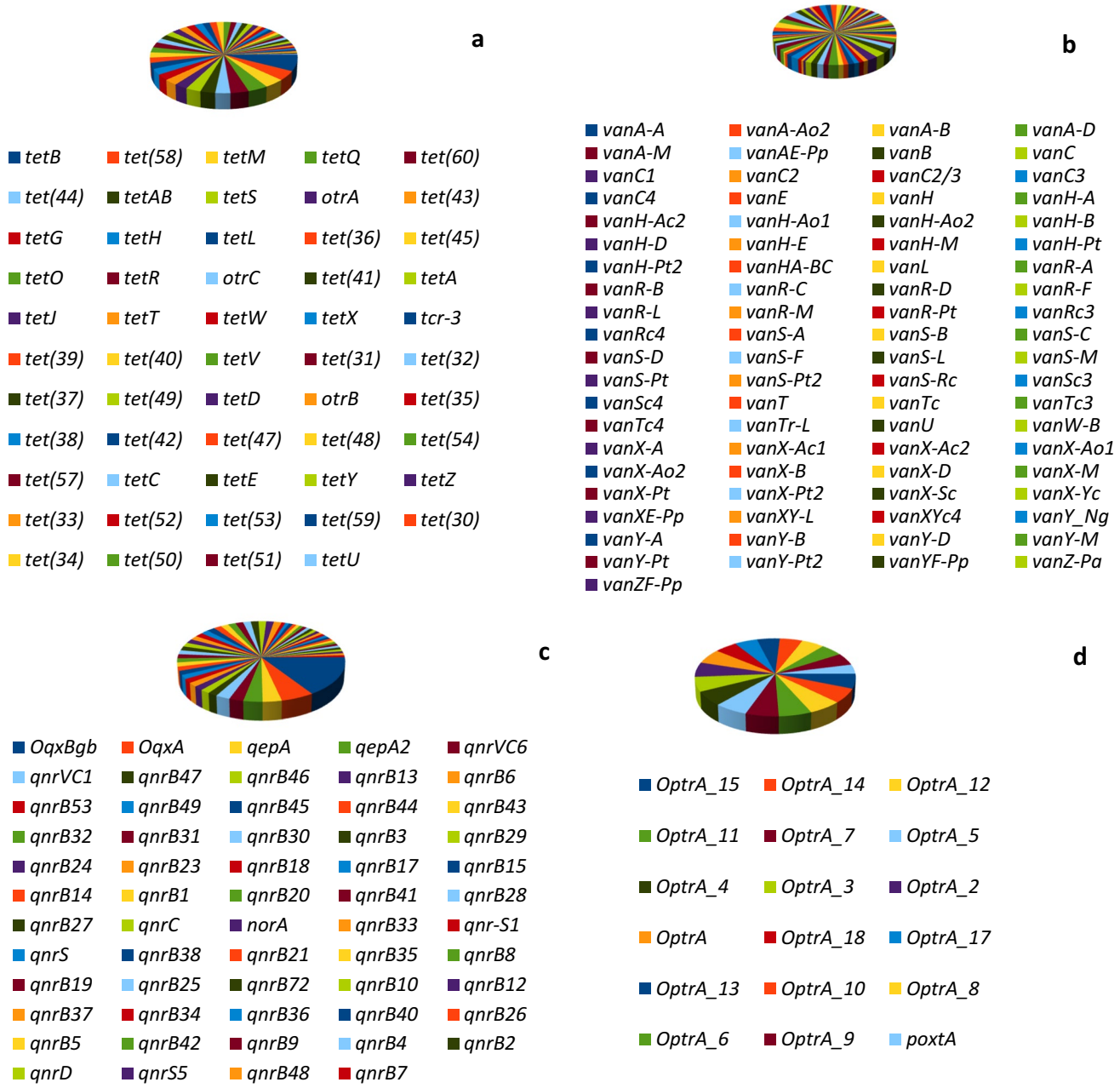

Fig. 4 Distribution of tetracycline (**a**), glycopeptide (**b**), fluoroquinolone (**c**) and oxazolidinone (**d**) resistance genes in 3S metagenome. Tetracycline resistance genes are classified based on their resistance mechanisms into ribosomal protection proteins (*otrA, tetM, tetO, tetS, tetT, tetW, tet(32), tet(36), tet(44), tetQ*), tetracycline-inactivating enzymes (*tet(34), tet(37), tet(47), tet(48), tet(49), tet(50), tet(51), tet(52), tet(53), tet(54), tetX*), and tetracycline efflux (*otrB, otrC, tetA, tetB, tetAB, tetC, tetD, tetE, tetG, tetH, tetJ, tetL, tetR, tcr-3, tet(30), tet(31), tet(33), tet(35), tet(38), tet(39), tet(40), tet(41), tet(42), tet(43), tet(45), tet(57), tet(58), tet(59), tet(60), tetU, tetV, tetY, tetZ*). Glycopeptide resistance genes either modify peptidoglycan precursors (*vanA, vanB, vanH, vanZ, vanC, van T, vanE, vanL*), hydrolyze normal peptidoglycan precursors (*vanX, vanY*), regulate expression of vancomycin resistance (*vanR, vanS*) or serve as transcriptional activator of vancomycin resistance (*vanU*). Fluoroquinolone resistance genes are dominated by *OqxBgb* and *OqxA* RND-type multidrug efflux pump and genes responsible for fluoroquinolone target protection (*qnrB, qnrC, qnrD, qnrS, qnrVC*). Oxazolidinone resistance genes is dominated by *OptrA* (and its variant), a member of the ABC-F ATP-binding cassette ribosomal protection protein

peptidoglycan precursors ending in D-Ala-D-Lac (*vanA, vanB, vanH, vanZ*, and their variants) or D-Ala-D-Ser (*vanC, van T, vanE, vanL* and their variants), hydrolysis of normal peptidoglycan precursors D-Ala-D-Ala (*vanX, vanY* and their variants), regulation of expression of vancomycin resistance (*vanR, vanS* and their variants), and transcriptional activator of vancomycin resistance (*vanU*). The gene *vanW* is an accessory gene found on vancomycin resistance operons with unknown function.

Fifty-nine (59) fluoroquinolone resistance genes were recovered from 3S metagenome (Fig. 4c). In terms of abundance, both the integral membrane protein (*OqxBgb*) and membrane fusion protein (*OqxA*) RND-type multidrug efflux pumps are dominant among the fluoroquinolone resistance genes. Other fluoroquinolone efflux pump detected is *qepA* gene, a major facilitator superfamily (MFS) antibiotic efflux pump. Other resistance genes detected are those responsible for fluoroquinolone target protection (*qnrB, qnrC, qnrD, qnrS, qnrVC* and their variants) and those responsible for increased expression of efflux pump (*norA*).

Eighteen (18) oxazolidinone resistance genes were retrieved from 3S metagenome (Fig. 4d). The resistance genes were dominated by *OptrA* (and its variant), a member of the ABC-F ATP-binding cassette ribosomal protection protein. The other resistance gene detected is *poxtA*, also a member of the ABC-F ATP-binding cassette ribosomal protection protein that confers resistance to tetracycline, phenicol and oxazolidinone via modification of the bacterial ribosome.

Trimethoprim, Nitroimidazole, Fusidic acid, and Sulfonamide resistance genes.

Thirty-six (36) trimethoprim resistance genes dominated by the *dfr* genes responsible for the modification of trimethoprim target enzyme dihydrofolate reductase (*dfr*) were recovered from 3S metagenome (Fig. 5). Of these, twenty-three (23) belong to the *dfrA* group constituting 63.9% of the trimethoprim resistance genes in the metagenome.

Six (6) nitroimidazole (nim) genes were recovered from 3S metagenome. These are *nimA* (3), *nimC* (1), *nimI* (3), *nimE* (1), *nimD* (1), and *nimJ* (1). Functional annotation using CARD detected a nitroimidazole resistance gene *msbA*, an ATP-binding cassette (ABC) antibiotic efflux pump not detected by ARG-ANNOT (Table 1). Only four (4) fusidic acid resistance genes *fusB* (1), *far1* (1), *fusH* (2), *fusD* (3) were detected in 3S metagenome. For sulfonamide resistance genes, four genes designated *sul1* (1), *sul2* (3), *sul3* (5) and *sul4* (4) were recovered in 3S metagenome.

Functional annotation of 3S metagenome using CARD revealed several resistance genes not detected by ARG-ANNOT. These include *arlR*, a major facilitator superfamily (MFS) antibiotic efflux pump that confer resistance to fluoroquinolones and acridine dye; *TaeA*, an ATP-binding cassette (ABC) antibiotic efflux pump that confer resistance to pleuromutilin antibiotic; and *MuxB*, a resistance-nodulation-cell division (RND) antibiotic efflux pump that confer resistance to macrolide, monobactam, tetracycline, and aminocoumarin antibiotics. Others include *golS* (monobactam, carbapenem, cephalosporin, cephamycin, penam, phenicol, penem); *evgS* (macrolide, fluoroquinolone, penam, and tetracycline antibiotics); *mdtA, mdtC* (aminocoumarin antibiotic) and several others (Table 1).

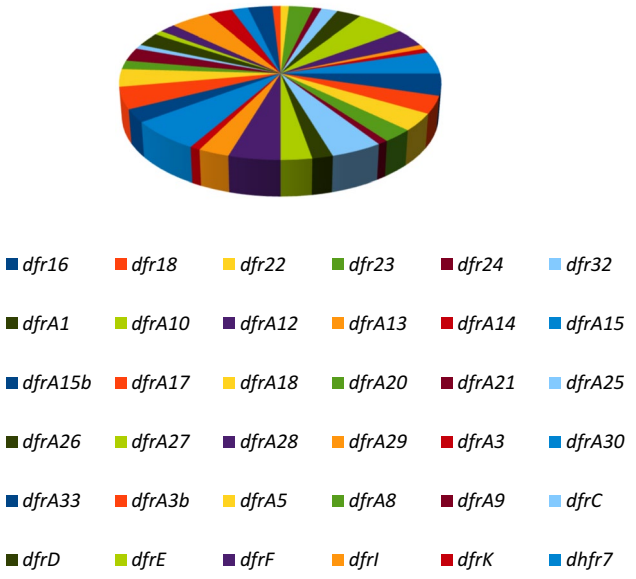

Fig. 5 Distribution of trimethoprim resistance genes in 3S metagenome. The resistance genes are dominated by the *dfr* genes responsible for the modification of trimethoprim target enzyme dihydrofolate reductase (*dfr*)

Heavy metal resistance genes

Copper (Cu) and silver (Ag) resistance genes

In 3S metagenome, copper resistance genes are represented by several systems (Table 2). These include the *cop* (*copA, copB, copC, copD, copS, copR, copY, copZ, copJ, copP, copL*), *cut* (*cutA, cutC, cutE, cutO*), *cue* (*cueA, cueR, cueO*), *cus* (*cusA, cusB, cusS*), *cme* (*cmeB. CmeC*) and the *pco* (*pcoA, pcoS*) systems as well as several transcriptional regulators (*copR, cueR, copY, hmrR, crdR, baeR, yfmP*) and transcriptional repressor (*csoR*). Also detected is a copper-exporting P-type ATPase *ctpV* gene and a gold/copper-translocating P-type ATPase *golT*. It is noteworthy that the genes *cueA* and *cusS* have dual resistance to copper and silver, respectively (Table 2).

Table 2 Heavy metal resistome detected in 3S metagenome (using BacMet) involved in resistance to heavy metals

Heavy metal	Enzymes/gene	Taxonomic affiliation
Copper, zinc, silver Iron, zinc, cobalt, nickel, copper, cadmium, Manganese, gallium	Copper resistance D; apolipoprotein N-acyltransferase actA (Cu/Zn); copper translocating P-type ATPase copB; cutE gene product; multicopper oxidase type 2 cutO; RND efflux system, inner membrane protein cmeB (Cu/Co); heavy metal translocating P-type ATPase copA; copper resistance protein copC; CopS protein; winged helix family two component heavy metal response transcriptional regulator copR; copper-sensing transcriptional repressor csoR; copper-translocating P-type ATPase cueA; MerR family transcriptional regulator cueR; metal-transporting P-type ATPase cueA (Cu/Ag); CutA1 divalent ion tolerance protein cutA; copper-binding protein cinA; RND efflux system, outer membrane protein cmeC (Cu/Co); multicopper oxidase type 3 cutO; molecular chaperone dnaK; transcriptional regulator copY; Cu(I)-responsive transcriptional regulator hmrR (Cu/HCl); Copper homeostasis protein cutC; Copper resistance protein L copL; laccase cueO; metal cation transporter P-type ATPase ctpV; heavy metal-translocating P-type ATPase actP; cation efflux system protein CusA; HAE1 family transporter acrD (Cu/Zn); sensor kinase cusS (Cu/Ag); thiol:disulphide interchange protein dsbC; integral membrane sensor signal transduction histidine kinase baeS (Cu/Zn/W); heavy metal translocating P-type ATPase golT (Cu/Au); response regulator crdR; type IIS restriction enzyme R protein crdS; copper resistance protein oxidase pcoA (plasmid); heavy metal sensor kinase pcoS (plasmid); CopJ protein (plasmid); heavy metal translocating P-type ATPase copZ; cation efflux system protein cusB (plasmid); winged helix family two component transcriptional regulator baeR (Cu/Zn/W); Heavy metal-translocating P-type ATPase Cd/Co/Hg/Pb/Zn-transporting ctpG (Cu); sensor histidine kinase corS; copper exporting ATPase copP; transcriptional regulator yfmP; crdB gene Zinc/iron ZIP family permease zupT (Zn/Fe/Co/Ni/Cu/Cd); iron-dependent repressor DxtR ideR; aconitase acn (Fe); transcriptional regulatory protein basR/pmrA (Fe); ferritin pfr (Fe/Cu/Mn); iron (III) ABC transporter, ATP binding protein fbpC (Fe/Ga); TonB-dependent siderophore receptor fpvA (Mn/Fe/Co/Zn/Ni/Cu/Cd/Ga); ABC transporter family protein ybtQ (Fe); cation efflux family protein fieF (Fe/Zn/Co/Cd/Ni); $Fe^{2+}/Zn^{2+}/Mn^{2+}$ ABC transporter ATP-binding protein troB; iron chelate ABC transporter, ATP-binding protein yfeB (Fe/Mn); Fe^{3+} ABC superfamily ATP-binding cassette transporter, permease protein fbpB (Fe/Ga); manganese transport protein mntH (Mn/Fe/Cd/Co/Zn); permease and ATP-binding protein of yersiniabactin-iron ABC transporter ybtP (Fe); Mn^{2+}/Fe^{2+} transporter NRAMP family (mntH/yfeB); TonB-dependent siderophore receptor fptA (Fe/Co/Ni/Ga); ferric uptake regulator family protein furA (Fe); cation-activated repressor protein troR (Zn/Mn/Fe); $Fe^{2+}/Zn^{2+}/Mn^{2+}$ ABC transporter membrane protein troD; ferritin Dps family protein dpsA (Fe/H_2O_2); chealated iron ABC transporter, permease yfeC (Fe/Mn); bacterioferritin bfrA (Fe); periplasmic iron-binding protein yfeA (Fe/Mn); putative ABC transport system permease fetB (Fe); iron transport inner membrane protein sitC (plasmid) ($Fe/Mn/H_2O_2$); fur regulated iron transporter sitB (plasmid) ($Fe/Mn/H_2O_2$); ferroxidase pfr (Fe/Cu/Mn); Chain A, Periplasmic Zinc Binding Protein troA (Zn/Mn/Fe); cation transport P-type ATPase nia (plasmid) (Fe/Ni)	*Rhodopseudomonas palustris* BisB18; alphaproteobacterium BAL199; *Methylobacterium* sp. 4–46; *Acidithiobacillus ferrooxidans* ATCC 53993; *Xenorhabdus bovienii* SS-2004; *Starkeya novella* DSM 506; *Campylobacter coli* JV20; *Paenibacillus dendritiformis* C454; *Thermobacillus composti* KWC4; *Ralstonia metallidurans* CH34; *Ralstonia pickettii* 12J; *Clostridium* sp. M62/1; *Pseudomonas syringae* pv. Tomato NCPPB 1108; *Pseudomonas mendocina* ymp; *Pseudomonas stutzeri* A1501; *Dickeya didantii* Ech586; *Marinobacter* sp. Mn17-9; *Campylobacter upsaliensis* JV21; *Streptomyces griseus* XylebKG-1; *Corynebacterium resistens* DSM 45100; *Oenococcus oeni* ATCC BAA-1163; *Pseudovibrio* sp. JE062; *Yersinia mollaretii* ATCC 43969; *Xanthomonas vesicatoria* ATCC 35937; *Aeromonas hydrophila*; *Mycobacterium marinum* M; *Beijerinckia indica* subsp. indica ATCC 9039; *Citrobacter youngae* ATCC 29220; *Pantoea stewartii* subsp. stewartii DC283; *Escherichia* sp. TW09308; *Enterococcus hirae*; *Photobacterium leiognathi* subsp. mandapamensis svers.1.1; *Glaciecola* sp. 4H-3-7+YE-5; *Agrobacterium tumefaciens* F2; *Helicobacter pylori* 35A; *Salmonella enterica* ser.. *Typhimurium*; *Helicobacter pylori* 2017; *Serratia marcescens*; *Ralstonia metallidurans* CH34; *Paenibacillus elgii* B69; *Escherichia coli* STEC_DG131-3; *Stenotrophomonas maltophilia* R551-3; *Saccharomonospora viridis* DSM 43017; *Myxococcus xanthus* DK 1622; *Streptococcus* sp. C150; *Bacillus pumilus* SAFR-032 *Methanosaeta harundinacea* 6Ac; *Corynebacterium accolens* ATCC 49725; *Corynebacterium nuruki* S6-4; *Pantoea vagans* C9-1; *Vibrio fischeri* MJ11; *Pseudomonas mendocina* NK-1; *Escherichia coli* DEC10F; *Shewanella benthica* KT99; *Treponema paraluiscuniculi* Cuniculi A; *Providencia rettgeri* DSM 1131; *Aggregatibacter segnis* ATCC 33393; *Pantoea* sp. SL1_M5; *Ralstonia solanacearum* CFBP2957; *Rhodobacter* sp. SW2; *Mycobacterium gilvum* PYR-GCK; *Treponema pallidum* subsp. pallidum str. Nichols; *Thioalkalimicrobium cyclium* ALM1; *Actinobacillus minor* NM305; *Rhodococcus pyridinivorans* AK37; *Pasteurella multocida* 36950; *Enterobacter cloacae* subsp. cloacae ATCC 13047; *Salmonella bongori* NCTC 12419; *Citrobacter rodentium* ICC168; *Cyanothece* sp. PCC 7425; *Treponema pallidum*; *Sinorhizobium meliloti* 1021

Table 2 (continued)

Heavy metal	Enzymes/gene	Taxonomic affiliation
Zinc, cadmium, tellurium, tungsten, cobalt, chromium nickel, manganese	BaeS gene product (Zn/W); Zn/Cd/Hg/Pb-transporting ATPase zntA (Zn/Pb/Cd); DNA-binding response regulator irlR (Cd/Zn); winged helix two component transcriptional regulator baeR (Zn/W); inorganic phosphate transporter pitA (Zn/Te); integral membrane sensor signal transduction histidine kinase actS (Cd/Zn/HCl); acriflavin resistance protein mdtB (Zn); zinc transporter zitB; high-affinity zinc transporter ATPase znuC (Zn); Zn(II)-responsive transcriptional regulator zntR; multidrug resistance transporter mdrL (Zn/Co/Cr); probable RND efflux transporter mdtB (Zn); membrane fusion protein (MFP-RND) heavy metal cation tricomponent efflux zneB/hmxB (plasmid) (Zn); high-affinity zinc transporter periplasmic component znuA (Zn); sensor protein zraS (Zn/Pb); zinc/iron permease zipB (Cd/Zn); heavy metal-translocating P-type ATPase ctpC (Mn/Zn); zinc efflux system zitB (Zn); outer membrane efflux protein czcC (plasmid; Cd/Zn/Co); cation transporting ATPase, P-type ziaA (Zn); response regulator receiver protein actR (Cd/Zn/HCl); RND family efflux transporter MFP subunit 1 mdtA (Zn); transcriptional regulator zur (Zn); thiol:disulfide interchange protein dsbA (Cd/Zn/Hg); transcriptional regulatory protein zraR (Zn); AcrB/AcrD/AcrF family protein nczA (Ni/Co/Zn); RND divalent metal cation efflux transporter czrA (Zn/Cd); cadmium-zinc-nickel resistance protein cznA (Cd/Zn/Ni); cadmium-translocating P-type ATPase czcP (Cd/Zn/Co); heavy metal response regulator irlR (Cd/Zn); dsbA-like protein frnE (Cd/H_2O_2); twin-arginine translocation pathway signal mntA (Cd/Mn); MerR family transcriptional regulator cadR (plasmid, Cd)	*Edwardsiella tarda* EIB202; *Pectobacterium carotovorum* subsp. brasiliensis PBR1692; *Burkholderia oklahomensis* EO147; *Geobacter* sp. M18; *Achromobacter piechaudii* ATCC 43553; *Sinorhizobium meliloti* CCNWSX0020; *Ralstonia pickettii* 12J; *Salmonella bongori* NCTC 12419; *Yokenella regensburgei* ATCC 43003; *Edwardsiella tarda* ATCC 23685; *Listeria monocytogenes* J0161; *Pseudomonas aeruginosa* NCMG1179; *Cupriavidus metallidurans* CH34; *Plautia stali* symbiont; *Escherichia coli* IAI39; *Pseudomonas* sp. TJI-51; *Kineococcus radiotolerans* SRS30216; *Pantoea stewartii* subsp. stewartii DC283; *Pyrococcus abyssi* GE5; *Rhizobium* sp. PDO-076; *Achromobacter arsenitoxydans* SY8; *Methylococcus capsulatus* str. Bath; *Aggregatibacter segnis* ATCC 33393; *Klebsiella pneumoniae* 342; *Caulobacter crescentus* CB15; *Pseudomonas aeruginosa* PA7; *Helicobacter mustelae* 12198; *Alicycliphilus denitrificans* BC; *Dermacoccus* sp. Ellin185; *Silicibacter* sp. TrichCH4B; *Pseudomonas putida* S16
Chromium, tellurium, selenium, molybdenum, vanadium, tungsten	ATP-dependent DNA helicase recG (Cr/Te/Se); Cr (VI) reductase (Cr, Fe, H_2O_2); NADPH-dependent FMN reductase chrR (Cr); putative NADH-dependent reductase yieF (Cr/V/Mo); malate dehydrogenase ruvB (Cr); chromate transporter chrA; nitroreductase A nfsA (Cr); NAD(P)H dehydrogenase (quinone) chrR (Cr); chromate resistance signal peptide protein chrB; Holliday junction DNA helicase ruvB (Cr/Te/Se); manganese/iron superoxide dismutase chrC (Cr); tellurite resistance protein tehB; tellurite resistance protein tehA; tellurite resistance protein trgB (Te/Cu); symporter actP (Te); KlaC protein klaC/telB (plasmid) (Te); molybdate ABC transporter inner membrane subunit modB (W/Mo); molybdate ABC transporter periplasmic protein wtpA (W/Mo); molybdenum ABC transporter ATP-binding protein modC (W/Mo); DNA-binding transcriptional regulator ModE (W/Mo); molybdenum ABC transporter periplasmic molybdate-binding protein modA (W/Mo); tungstate ABC transporter binding protein wtpA (W/Mo); ABC transporter, ATP-binding protein wtpC (W/Mo); molybdate/tungstate transport system permease protein wtpB; molybdenum-pterin binding domain protein/site-specific recombinase, phage integrase family tunR (W/Mo); sodA gene product (Se/H_2O_2); superoxide dismutase sodB (Se/H_2O_2)	*Tolumonas auensis* DSM 9187; *Lysinibacillus fusiformis* ZC1; *Burkholderia multivorans* ATCC 17616; *Burkholderia cepacia*; *Shewanella frigidimarina* NCIMB 400; *Sinorhizobium meliloti* BL225C; *Vibrio vulnificus* YJ016; *Rhodobacter capsulatus* SB 1003; *Acinetobacter oleivorans* DR1; *Alkalilimnicola erhlichii* MLHE-1; *Alicycliphilus denitrificans* K601; *Haemophilus pittmaniae* HK 85; *Haemophilus haemolyticus* M21621; *Maritimibacter alkaliphilus* HTCC2654; *Magnetospirillum magneticum* AMB-1; *Ralstonia pickettii* 12J; *Desulfotomaculum kuznetsovii* DSM 6115; *Gillisia limnaea* DSM 15749; *Salmonella enterica* subsp. enterica serovar Typhimurium str. T000240; *Desulfotomaculum acetoxidans* DSM 771; *Methanolinea tarda* NOBI-1; *Thermococcus sibiricus* MM 739; *Thermococcus litoralis* DSM 5473; *Desulfovibrio vulgaris* str. Hildenborough; Serratia symbiotica str. 'Cinara cedri'; *Burkholderia pseudomallei* 1655

Table 2 (continued)

Heavy metal	Enzymes/gene	Taxonomic affiliation
Nickel, cobalt, manganese, magnesium	Major facilitator transporter nrsD/nreB (Ni/Co); P-ATPase superfamily, P-type ATPase cadmium transporter ctpD (Co/Ni); cation transporter dmeF (Co/Ni); magnesium and cobalt transport protein corA (Mg/Co/Ni); nickel ABC transporter substrate-binding protein nikA (Ni); iron-dicitrate transporter subunit, membrane component of ABC superfamily fecD (Ni/Co); nickel and cobalt resistance protein cnrA (Co/Ni); nickel transporter ATP-binding protein nikE (Ni); nickel ABC superfamily ATP binding cassette transporter, ABC protein nikD (Ni); integral membrane sensor signal transduction histidine kinase nrsS (Ni); high-affinity nickel transporter rcnA (Ni/Co/Fe); fecE gene product (Ni/Co); periplasmic nickel sensor cnrR/cnrX (plasmid, Ni); RNA polymerase sigma factor cnrH (plasmid, Co/Ni); nickel ABC transporter, permease subunit nikC (Ni); transcriptional regulator, copG family protein nikR (Ni); nickel ABC transporter permease nikB; czcA family heavy metal efflux pump nrsA (Ni); nickel/cobalt efflux system ncrC (Co/Ni); RNA polymerase sigma factor nccH (plasmid; Ni/Co/Cd); ArsR family transcriptional regulator cmtR (Co/Ni); conserved inner membrane protein mntP (Mn/Mg); magnesium-transporting ATPase P-type 1 mgtA; CO^{2+}/Mg^{2+} efflux protein ApaG corD (Co/Mg); magnesium and cobalt efflux protein corB; Mg/Co transporter corC; magnesium/nickel/cobalt transporter corA	*Roseiflexus* sp. RS-1; *Mycobacterium parascrofulaceum* ATCC BAA-614; *Pelobacter carbinolicus* DSM 2380; *Alkanivorax* sp. DG881; *Dickeya dadantii* Ech703; *Escherichia coli* S88; *Bradyrhizobium* sp. STM 3843; *Rhodospirillum rubrum* ATCC 11170; *Roseomonas cervicalis* ATCC 49957; *Cyanothece* PCC 7424; *Starkeya novella* DSM 506; *Xenorhabdus nematophila* ATCC 19061; *Cupriavidus metallidurans* CH34; *Brucella* sp. NF 2653; *Sagittula stellata* E-37; *Desulfotomaculum kuznetsovii* DSM 6115; *Klebsiella oxytoca* 10-5245; *Clavibacter michiganensis* subsp. sepedonicus; *Pantoea stewartii* subsp. stewartii DC283; *Klebsiella oxytoca* 10-5246; *Pseudomonas psychrotolerans* L19; *Vibrio* sp. Ex25; *Idiomarina loihensis* L2TR; *Actinobacillus succinogenes* 130Z
Mercury, lead, arsenic, antimony	Mercuric reductase merA; periplasmic mercury transport protein merT; alkylmercury lyase merB2; MerR family transcriptional regulator merR1; heavy metal transport/detoxification protein merP; copper-transporting ATPase merT-P; putative mercury transport protein merC (plasmid); Pb-efflux ATPase pbrA; Pb-specific transcription regulator protein pbrR (plasmid); iron permease FTR1 family protein 2 pbrT (plasmid); phosphate import ATP binding protein pstB (As); arsenical resistance protein arsB (As); arsenical resistance protein acr3 (As); arsenite efflux pump ACR3 arsB (As/Sb); arsenate reductase arsC (As/Sb); molydopterin dinucleotide binding region (As); methyltransferase type 11 arsM; phosphate-binding protein pstS (As); phosphate ABC transporter permease protein pstC (As); MIP family channel protein glpF (As/Sb); phosphate transporter permease subunit A pstA (As); arsenical pump-driving ATPase arsA (plasmid, As/Sb); putative arsenic-efflux pump regulatory protein arsR (As/Sb/Bi); putative sensor histidine kinase aioS/aoxS (As); arsenite oxidase small subunit (As); arsenite oxidase large subunit	*Micromonospora* sp. L5; *Thiobacillus* sp.; *Nocardioides* sp. JS614; *Xanthobacter autotrophicus* Py2; *Croceibacter atlanticus* HTCC2559; *Acidithiobacillus ferrooxidans* ATCC 53993; *Ralstonia metallidurans* CH34; *Herbaspirillum seropedicae* SmR1; *Achromobacter xylosoxidans* A8; *Sutterella wadsworthensis* 3_1_45B; *Moorella thermoacetica* ATCC 39073; *Campylobacter lari*; *Synechococcus* sp. PCC 7002; *Lactobacillus reuteri* SD2112; *Pyrobaculum calidifontis* JCM 11548; *Intrasporangium calvum* DSM 43043; *Cupriavidus basiliensis* OR16; *Nitrococcus mobilis* Nb-231; *Pseudomonas fulva* 12-X; *Leptothrix cholodnii* SP-6; *Klebsiella pneumoniae* 342; *Agrobacterium tumefaciens*; *Burkholderia multivorans* ATCC 17616; *Roseovarius* sp. 217

Iron (Fe), manganese (Mn), and gallium (Ga) resistance genes

Majority of resistance genes detected for iron in 3S metagenome are genes that also confers resistance on other heavy metals such as zinc, cobalt, nickel, copper, cadmium, manganese and gallium (Table 2). These genes include *zupT, pfr, fbpC, fpvA, fieF, yfeB, troB, troA, sitB, sitC*, and several others. However, specific genes that confer resistance on iron alone such as *ideR, ybtP, ybtQ, furA, bfrA, fetB*, and several others were also detected. All the resistance genes detected for manganese and gallium confers resistance on other heavy metals. These include f*bpC, fbpB, yfeB, mntH, mntA, troD, fptA, yfeA* and several others (Table 2).

Zinc (Zn), cadmium (Cd), nickel (Ni), and cobalt (Co) resistance genes

Several zinc resistance genes were recovered from 3S metagenome (Table 2). These include *zitB, znuC, mdtB, zntR, zneB/hmxB, znuA, ziaA, zur, zraR* and several others. Resistance genes that confer resistance on zinc and other heavy metals such as *zntA* (Zn/Pb/Cd), *pitA* (Zn/Te), *mdrL* (Zn/Co/Cr), *zipB* (Zn/Cd) among others were also detected. Cadmium resistance genes such as *czcC* (Cd/Zn/Co), *czrA* (Cd/Zn), *cznA* (Cd/Zn/Ni), *czcP* (Cd/Zn/Co), *cadR*, among others, which confers resistance on cadmium and other heavy metals were also detected (Table 2).

In addition, representatives of nickel resistance genes (*nikA, nikB, nikC, nikD, nikE, nrsA, nrsS*, among others) and those that confer resistance to nickel and other heavy metals (*nczA* (Ni/Co/Zn), *nia* (Ni/Fe), *nrsD/nreB* (Ni/Co), *dmeF* (Co/Ni), *fecD* (Ni/Co), *cnrA* (Co/Ni), *rcnA* (Ni/Co/Fe)) and several others were also recovered from 3S metagenome (Table 2).

Chromium (Cr), tellurium (Te), selenium (Se), molybdenum (Mo), tungsten (W) and vanadium (V) resistance genes

In 3S metagenome, several chromium resistance genes were recovered. These include Cr (IV) reductase, *chrA, chrB, chrC, chrR, nfsA, yieF* (Cr/V/Mo), *ruvB* (Cr/Te/Se), *recG* (Cr/Te/Se) among others. Tellurium resistance genes detected include *tehA, tehB, trgB* (Te/Cu), and *klaC/telB*, respectively. Representatives of Molybdenum and tungsten resistance genes detected include *modA* (W/Mo), *modB* (mO/W), *modC* (W/Mo), *modE* (W/Mo), *wtpA* (W/Mo), *wtpC* (W/Mo) among others while *sodA* and *sodB* genes were recovered for selenium resistance (Table 2).

Magnesium (Mg), mercury (Hg), lead (Pb), arsenic (As), antimony (Sb) resistance genes

Functional annotation of 3S metagenome revealed the following genes responsible for magnesium, mercury, lead, aresenic and antimony resistance: Mg (*mntP, mgtA, corA, corB, corC, corD*); Hg (*merA, merT, merB2, merR1, merP, merT-P, merC*); Pb (*pbrA, pbrR, pbrT*); As/Sb (*pstA, pstB, pstC, pstS, arsA, arsB, acr3, arsC, arsM, arsR, glpF*, arsenite oxidase small/large subunits) (Table 2).

Discussion

Soil is a principal member of the ecosystem. Its inundation with diverse pollutants such as antibiotics and heavy metals from diverse sources has resulted in the proliferation of antibiotic and heavy metal resistance genes in the soil ecosystem with attendant consequences on public and environmental health. In this study, an attempt was made to unravel the antibiotic and heavy metal resistome in a chronically polluted soil using a shotgun metagenomic approach. This study is important for several reasons. First, the alarming rate and ease of dissemination and spread of antibiotic resistance genes between environmental compartments are disturbing, as several authors have indicated that the soil resistome may be a critical source for the emergence of novel antibiotic resistance genes in clinical pathogens (Willms et al. 2019; Zhu et al. 2019). Second, it is widely believed that pristine soil microbiomes comprise a wide array of novel and clinically characterized native antibiotic resistance genes, whose presence predates clinical use of antibiotics (Perry and Wright 2013; Durso et al. 2016). Third, co-selection of antibiotic and heavy metal resistance in microorganisms via cross-resistance, co-resistance and co-regulatory mechanisms has maintained and promote antibiotic resistance in microbial populations in the absence of antibiotics (Pal et al. 2015a, b).

β-lactamases, produced extracellularly by Gram-positive bacteria and in the periplasmic space by Gram-negative bacteria inactivate β-lactam antibiotics by binding covalently to their carbonyl moiety and hydrolyzing the β-lactam ring. Based on Ambler class A-D classification, Ambler classes A, C, and D are β-lactamases with serine at their active site while Ambler class B β-lactamases are metallo-enzymes that require zinc as a metal cofactor for their catalytic activities (van Hoek et al. 2011). In this study, 144 β-lactamase genes were recovered. Of these, 60 (41.7%) belong to class A β-lactamases. This is concerning as most of the extended-spectrum β-lactamases (ESBLs) belong to this class (Eiamphungporn et al. 2018). ESBLs, which are plasmid-borne are reputed for their ability to hydrolyze third and fourth generation cephalosporins and monobactams but not cephamycins

(e.g. cefoxitin) and carbapenems (meropenem, imipenem, ertapenem, and doripenem) and are generally susceptible to β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Paterson and Bonomo 2005; Eiamphungporn et al. 2018; Rahman et al. 2018).

Expectedly, though with a narrow margin, the number of variants of blaCTX-M (cefotaximase) genes detected in this study trumped other *Enterobacteriaceae* ESBL enzymes blaTEM and blaSHV. This may be attributed to extraordinary dissemination of blaCTX-M genes in highly mobilizable plasmids and transposons and the phenomenon of co-resistance especially to aminoglycosides and fluoroquinolones synonymous with blaCTX-M gene-carrying microorganisms, which might facilitate co-selection processes (Canton and Ruiz-Garbajosa 2011; Rogers et al. 2011). blaCTX-M β-lactamases exhibit high hydrolytic activity against cefotaxime and a higher hydrolytic activity on cephalothin when compared to penicillins, cefaloxine and ceftazidime (Tzouvelekis et al. 2000). The second dominant class A β-lactamase in this study, blaTEM, the first plasmid-mediated β-lactamase is the most prevalent plasmid-encoded β-lactamase in Gram-negative bacteria responsible for about 90% of their resistance to ampicillin (van Hoek et al. 2011; Rahman et al. 2018). Its classic phenotype blaTEM-1 exhibits hydrolytic activity on penicillin and first-generation cephalosporin such as cephaloridine. Also detected are class A β-lactamases with carbapenem-hydrolyzing activity such as blaKPC, blaGES, and blaIMI. However, of great concern is the detection of blaKPC, as KPC β-lactamases are known to efficiently hydrolyze carbapenems, penicillins, cephalosporins and aztreonam. They also hydrolyze clinically available β-lactamase inhibitors (with the exception of azobactam, which hydrolyze KPC enzymes slowly) such as clavulanic acid, sulbactam, and tazobactam (Nguyen et al. 2016; Bonomo 2017).

The most prominent representatives of class B metalloenzymes β-lactamases detected in 3S metagenome are blaNDM and blaVIM constituting 22.1% and 15.5% of the class B β-lactamases. This is not surprising, as NDM (New Delhi MBL), which have been reported from diverse geographical locations are not inhibited by commercially available β-lactamases, genes are borne on transferable plasmids, chromosome and integrons and have tight hydrolytic activities on penicillins, cephalosporins and carbapenems (Bonomo 2017). In addition, the promiscuous spread of blaNDM genes among bacterial species, particularly members of *Enterobacteriaceae* (Darley et al. 2012; Jones et al. 2014), the possibility of its environmental spread (Walsh et al. 2011), and its concomitant carriage of loads of resistance determinants for β-lactamases, carbapenemases, and genes associated with resistance to fluoroquinolones and aminoglycosides (Bonomo 2017) makes blaNDM a public and environmental health menace.

Plasmid-mediated AmpC β-lactamases such as blaCMY, blaLEN, blaOKP dominated the class C β-lactamases recovered from 3S metagenome. These plasmid-mediated enzymes confer resistance to a broad-spectrum of β-lactam antibiotics including oxyimino-β-cephalosporins, penicillins, cephamycins and variably aztreonam (Jacoby 2009). Previous reports have also indicated co-selection of multiple resistance genes by plasmids carrying AmpC β-lactamases, which confer resistance to aminoglycosides, chloramphenicol, sulfonamides, quinolones, tetracycline and trimethoprim as well as genes for other β-lactamases (Jacoby 2009; Rahman et al. 2018).

In this study, the predominant class D β-lactamase genes are the blaOXA genes constituting 91.7% of the class members. The preponderance of the class D OXA-type enzymes may be attributed to mutation resulting in amino acid substitutions and the emergence of a high number of variants with an extended and narrow spectrum of activities against diverse β-lactam antibiotics such as penicillins, cephalosporins, extended-spectrum cephalosporins and carbapenems (van Hoek et al. 2011; Leonard et al. 2013; Bonomo 2017). The genes for OXA-type enzymes are also borne on plasmids and integrons, which allow promiscuous dissemination and spread of the resistance across not only members of *Enterobacteriaceae* (Castanheira et al. 2011; Patel and Bonomo 2013) but also Gram-positive bacteria belonging to the *Bacillaceae*, *Clostridiaceae*, and *Eubacteriaceae* families of the phylum *Firmicutes* (Toth et al. 2016).

Four mechanisms of resistance to aminoglycoside antibiotics have been reported. They are active efflux, decrease permeability, ribosome alteration, and antibiotic inactivation by aminoglycoside-modifying enzymes (van Hoek et al. 2011). In this study, majority of the aminoglycoside resistance genes detected code for aminoglycoside-modifying enzymes. Prominent among them is *aadA* gene, streptomycin 3′-adenyltransferase, a member of the ANT (3″) family, which confer resistance to streptomycin and spectinomycin and is encoded by plasmids, transposons and integrons in *Enterobacteriaceae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* (Hollingshead and Vapnek 1985). Others include aac(6′) and aac(3) and their variants, which are plasmid-encoded aminoglycoside acetyltransferases that confers resistance to gentamicin, tobramycin, kanamycin (aac(3)), neomycin (aac(3)), amikacin (aac(6′)), and paromycin (aac(3)), respectively. These plasmid-encoded genes have been reported in *Klebsiella pneumoniae, Enterobater cloacae*, *Actinobacillus pleuropneumoniae, Salmonella typhimurium, Citrobacter freundii, Acinetobacter* spp., *Stenotrophomonas* spp., and *Pseudomonas aeruginosa* (Allmansberger et al. 1985; Shaw et al. 1993; Mugnier et al. 1996; Ramirez and Tolmansky 2010; van Hoek et al. 2011).

MLS antibiotics comprise three chemically distinct, but functionally similar antibiotic classes. However, due to

similar mode of antibacterial action of macrolide and the two other antibiotic classes, lincosamide and streptogramin B and their comparable antibacterial spectra, they have been clustered together as macrolide-lincosamide-streptogramin (MLS) antibiotics (van Hoek et al. 2011; Marosevic et al. 2017). Three main resistance mechanisms have been reported for MLS antibiotics. These are rRNA methylation, active efflux and antibiotic inactivation. In this study, rRNA methylation mediated by rRNA methylases encoded by *cfr; erm; tlr; myr; emt* genes is the dominant MLS resistance mechanism in 3S metagenome. This is disheartening for two reasons. First, rRNA methylases, particularly those encoded by *erm* genes confer cross-resistance between macrolides, lincosamides and streptogramin B antibiotics. Second, there are several reports detailing co-selection of tetracycline and MLS resistance genes (especially *erm*) on large transposons such as Tn1545 (tet(M), macrolide-aminoglycoside-streptothricin element, erm(B)) (Cochetti et al. 2008), and CTnDOT family transposons (tet(Q), tet(X), erm(B), erm(F), erm(G)) (Shoemaker et al. 2001; Gupta et al. 2003). Moreover, some of these transposons belong to the Tn916 family, which has a broad host range and transfer promiscuously to a wide variety of Gram-positive and Gram-negative bacteria (Roberts and Mullany 2011).

Colistin, extensively used in agricultural production and treatment of clinical pathogens in spite of its potential nephrotoxicity and neurotoxicity, is the ultimate line of defence against critical infections caused by multidrug-resistant pathogens (Xu et al. 2018). However, the emergence of mobile colistin resistance (*mcr*) genes is compromising the efficacy of colistin as last-line antibiotic used to treat multidrug-resistant Gram-negative bacteria. In this study, *mcr-3* and *mcr-1* are the most prominent *mcr* genes recovered from 3S metagenome. This is not surprising as several reports have indicated the preponderance of mcr-1 and mcr-3 in clinical specimens, clinical isolates, MDR lineages of *E. coli* and in livestocks (pigs, poultries, cows, goats as a growth promoter and to treat infections) across several continents (Catry et al. 2015; Gao et al. 2016; Hu et al. 2016; Ma et al. 2016; Bi et al. 2017; Coates et al. 2017; Haenni et al. 2017; Litrup et al. 2017). In addition, most of the mcr-3 genes and its variants are located on IncHI2-type plasmids, IncP-like plasmid, and IncF plasmid, which facilitate their spread and dissemination (Haenni et al. 2017). Open defecation and urination by humans and unregulated rearing of livestock and its attendant defecation and urination along rearing paths, a regular activity in the geographical region where this study is conducted could be fingered as possible routes for spread and dissemination of the *mcr* genes in the soil environment.

Chloramphenicol acetyltransferases encoded by the *cat* genes dominated the phenicol resistance genes recovered from 3S metagenome. These enzymes inactivate chloramphenicol, thiamphenicol and azidamfenicol by attaching an acetyl group from acetyl-CoA to the antibiotics, which prevent them from binding to the ribosome (Murray and Shaw 1997; Schwarz et al. 2004; Wright 2005). Diverse Gram-positive and Gram-negative bacteria species have been reported to encode the *cat* genes. These include *Acinetobacter calcoaceticus, Agrobacterium tumefaciens, Bacillus clausii, Bacillus subtilis, Campylobacter coli, Enterococcus faecalis, Enterococcus faecium, Lactococcus lactis, Listeria monocytogenes, Morganella morganii, Proteus mirabilis, Serratia marcescens, shigella flexneri, Staphylococcus aureus, S. haemolyticus, S. intermedius, Streptococcus agalactiae, S. suis, S. acrimycini* (Projan et al. 1985; Schwarz et al. 1991; Tennigkeit and Matzura 1991; Cardoso and Schwarz 1992; Perreten et al. 1997; Parkhill et al. 2001; Grady and Hayes 2003; Takamatsu et al. 2003; Galopin et al. 2009).

Tetracycline antibiotics, well known for their broad spectrum of activity, encompasses a wide range of Gram-positive and Gram-negative bacteria, spirochaetes, atypical organisms such as mycoplasmas, chlamydiae and rickettsiae, as well as protozoan parasites (Kobashi et al. 2006). The drug has proven clinical safety, acceptable tolerability, and it is available in intravenous and oral formulations for most members of the class (Grossman 2016). Extensive use of tetracyclines in veterinary medicine and agricultural applications, including crop protection and intensive animal farming, has contributed to the widespread dissemination of tetracycline resistance (Surette and Wright 2017; Wu et al. 2020). In this study, resistance genes encoding ribosomal protection proteins, tetracycline-inactivating enzymes, and tetracycline efflux were detected. However, more disturbing is the detection in this study, of seven (*tetX, tet*(*49*), *tet*(*50*), *tet*(*51*), *tet*(*52*), *tet*(*53*), *tet*(*54*)) out of the nine tetracycline destructases that mediate enzymatic inactivation of third (tigecycline) and fourth (eravacycline and omadacycline) generation tetracyclines known to overcome resistance due to efflux and ribosome protection (Jenner et al. 2013; Tanaka et al. 2016; Zhanel et al. 2016). The destructases mediate covalent destruction of the antibiotic scaffold via enzymatic inactivation, which permanently eliminates the tetracycline antibiotic challenge by lowering intracellular and extracellular antibiotic concentrations (Markley and Wencewicz 2018). Moreover, the genes encoding the destructases, which are often borne on transferable plasmids, are normally present on operons that are co-transcribed (to ensure self-protection) with biosynthetic genes in the antibiotic-producing microorganism (Davies and Davies 2010; Mack et al. 2014; Li et al. 2018).

Majority of fluoroquinolone resistance genes recovered in this study such as *OqxBgb, OqxA, qepA* and *qnr* are plasmid encoded, which facilitate their spread and dissemination via horizontal gene transfer (Hooper and Jacoby 2016).

OqxAB gene, first identified as a plasmid-mediated efflux pump conferring resistance to the olaquindox, also confer resistance to other quinolones ciprofloxacin and norfloxacin as well as other antibiotics such as chloramphenicol, nitrofurantoin, and trimethoprim (Hansen et al. 2007; Ho et al. 2016) and have been reported in multiple species of *Enterobacteriaceae*. Similarly, plasmid-borne *qepA* gene efflux pump, which has now been found worldwide (Habeeb et al. 2014; Zhao et al. 2015), have been reported in clinical *E. coli* isolates (often associated with aminoglycoside resistance) by researchers from Japan and France (Pe´richon et al. 2007; Yamane et al. 2007). In addition, *qnr* genes have been found regularly in multi-resistance plasmids that also encode genes for other resistance determinants such as β-lactamases, ESBLs, AmpC enzymes, and carbapenemases (Jacoby et al. 2014).

The detection of sulfonamide resistance genes *sul1-4* in the 3S metagenome is interesting because sulfonamides are synthetic antibiotics, and naturally occurring enzymes that degrade or modify this antibiotic are not to be expected. Thus, its detection indicates active pollution of the polluted soil with the antibiotic, its residual or antibiotic resistance bacteria carrying the *sul* gene. Mobile genetic elements facilitate the spread and dissemination of the *sul* gene via horizontal gene transfer. This is evident in the location of the *sul* gene. While *sul1* gene and other resistance determinants is located on class 1 integrons, *sul2* gene is located on IncQ-type non-conjugative plasmid and large transmissible plasmids, and *sul3* gene is reported to be encoded on *E. coli* conjugative plasmid pVP440 flanked by two copies of IS element IS15Δ/26 (Razavi et al. 2017). *sul4* gene is also believed to be mobile genetic element borne.

The preponderance of antibiotic resistance genes against β-lactam antibiotics as well as other antibiotics used to treat infection caused by members of the family *Enterobacteriaceae* at the sampling site point to possible direct/indirect contamination from faecal materials of medicated humans and animals from point and non-point sources.

While the presence of heavy metal resistance genes at the sampling site may be attributed to indiscriminate dumping of spent oils and other hydrocarbon products rich in heavy metals, which normally select for microorganisms with heavy metal resistance phenotype, various anthropogenic activities that predates this period (i.e. 15 years) may have also contributed to the soil heavy metal burden and the emergence of heavy metal resistance genes. In addition, protists are known to employ metal poisoning to inactivate their bacteria prey before killing them. To counteract this, bacteria have evolved diverse metal detoxification strategies such as copper/zinc resistance genes to avoid killing via metal poisoning (Hao et al. 2015, 2016, 2017). Thus, a non-anthropogenic scenario such as this also fuels the spread and dissemination of heavy metal resistance genes in bacteria.

Aside from being a constituent of spent oils and hydrocarbon products, copper have been used in aquaculture, horticulture and a pesticide or antimicrobial in livestock production. It is also added to animal feed as a growth promoter by influencing the gut microbiota (Pal et al. 2017). Bacterial cells have evolved systems for copper homeostasis to protect against excess copper-mediated toxicity while allowing the adequate supply of copper for cellular processes. In this study, four copper resistance systems *cue, cop, cus, and pco* used by members of the family *Enterobacteriaceae* were detected. In the *cue* system of *E. coli* used under both aerobic and anaerobic conditions (Bondarczuk and Piotrowska-Seget 2013), a MerR family copper (I)-responsive transcriptional activator, CueR, regulates expression of a copper efflux P1-type ATPase, CopA and of CueO, a periplasmic multi-copper oxidase that oxidize Cu(I) to Cu(II) (Hobman and Crossman 2014; Pal et al. 2017). Under anaerobic and extreme copper stress conditions, CueO is inactive due to oxygen limitation. As a result, *E. coli* use the *cus* system comprising a two-component regulator CusRS that activate expression of *cusCFBA* (in response to stress and elevated Cu(I) levels at the cell envelope); a tripartite RND family silver/copper efflux and CusF, a periplasmic metallochaperone (Munson et al. 2000; Outten et al. 2001). In the plasmid-borne copper resistance *pco* system, only PcoA, a multi-copper oxidase that oxidize Cu(I) to less toxic Cu(II) and PcoS, a member of the two-component regulator PcoRS, which regulate gene expression from the *pco* operon (Hobman and Crossman 2014) were detected in 3S metagenome. The detection of the Gram-positive *copYZAB* operon *cop* system (Pal et al. 2017); the Gram-negative *copABCDRS* operon *cop* system (Monchy et al. 2006; Zimmermann et al. 2012; Staehlin et al. 2016), *cinA* of *Pseudomonas putida* copper-induced cinAQ system; *crdB, crdR, crdS* of *Helicobacter pylori* CrdA-CrdB-CzcB-CzcA system; and *csoR, ctpV* of *Mycobacterium tuberculosis* (Waidner et al. 2005; Liu et al. 2007; Quaranta et al. 2009) and several others in 3S metagenome further highlight the diverse copper resistance systems employed by members of the microbial community for detoxification, transport and export of excess copper.

The detection of diverse resistance mechanisms for zinc, cadmium, cobalt, and nickel responsible for uptake, detoxification and efflux in 3S metagenome may be attributed to selective pressure imposed by the heavy metal burden on the microbial community, which naturally select for members of the microbial community with heavy metal resistance phenotypes. Resistance genes encoding for the transport of zinc and other divalent cations (zupT (Zn/Fe/Co/Ni/Cu/Cd), zipB (Zn/Cd) (Fox and Guerinot 1998), high-affinity zinc transporter znuA, znuC (Patzer and Hantke 1998), as well as nickel (nickel ABC transporter nikA, nikB, nikC, nikD, nikE and several others)) were detected. Also detected are genes responsible for efflux (ZntA (Zn/Cd/Pb), ZitB (Zn),

ncrC (Co/Ni), czrA (Zn/Cd; Hassan et al. 1999), czcA, czcB, czcC (Cd/Zn/Co; Nies et al. 1989; Franke et al. 2003; Nies 2003a, b) and several others), and transcriptional regulation (zntR, zraR, zraS, cadR, cmtR) among others (Table 2).

Of great concern is the detection of *mer* resistance genes, which are usually borne on *mer* operon in 3S metagenome. In Hg^{2+} detoxification, periplasmic Hg^{2+}-binding protein MerP bound to the Hg^{2+} and delivers it to the mercury transporter MerT for transport into the cytoplasm, where it is reduced in a NADPH-dependent manner to volatile, less reactive Hg^0 by MerA, mercuric reductase (Nies 1999; Oregaard and Sorensen 2007). In this study all the genes encoding MerP, MerT, MerA (*merP, merT, merA*) were detected in 3S metagenome (Table 2). More so, resistance gene for alternative route of mercury uptake *merC* (Hamlett et al. 1992; Sahlman et al. 1997) was also detected in this study, thus, indicating that some members of the microbial community also use the alternative route for mercury uptake. In addition, as shown in Table 2, resistance to lead (Pb) in 3S metagenome is mediated a P-type ATPase, Pb-efflux ATPase pbrA, which facilitate effective efflux of Pb^{2+} from the cytoplasm.

Arsenic toxicity stem from its structural similarity to phosphate, which allows it to interfere in the metabolism of phosphorus, a major macronutrient required by microorganisms. Arsenate As(V) enters the cell via the phosphate uptake systems (PstA, PstB, PstC) where it is reduced to arsenite As(III) by arsenate reductase ArsC to facilitate its differentiation from phosphate. As(III) can also be taken up directly using GlpF, a MIP (Major Intrinsic Protein) family channel protein. The resultant As(III) is thereafter translocated across the cytoplasmic membrane via ArsB or Acr3 (Nies 1999; Ben Fekih et al. 2018). It is instructive to note that genes encoding PstA, PstB, PstC (*pstA, pstB, pstC*) as well as ArsC, ArsB, Acr3, GlpF (*arsC, arsB, acr3, glpF*) and several others were detected in the metagenome. ArsA, which catalyse the extrusion of arsenite (Wang et al. 2004); arsenite oxidase, which mediates the oxidation of toxic arsenite to less toxic arsenate (Ellis et al. 2001); and ArsM, which is involved in the conversion of As(III) to a number of methylated products (Wang et al. 2014) were also detected in this study (Table 2).

The mercury *mer* operon are usually borne on integrons, which of recent are found to be carried on transposons such as *Tn21*. The *Tn21* transposons carries *In2* integrons inserted between the mercury resistance genes and the transposition genes, which also carries the integrase gene *intI1* that can acquire, express and reassort the antibiotic resistance cassettes carried by the integrons (Rosewarne et al. 2010; Mindlin and Petrova 2013). The consistent link between mercury resistance and antibiotic resistance in microorganisms from diverse habitats such as faecal and oral microbiota of primates (Wireman et al. 1997), fish gastrointestinal tracts (Akinbowale et al. 2007), mine sediments (Nemergut et al. 2004), and freshwater microcosms (Stepanauskas et al. 2006) is a cause for serious public health concerns and this has been largely fuelled by the presence of integron-associated integrases with antibiotics and heavy metal resistance determinants in mercury transposons (Pal et al. 2015a, b).

The co-resistance phenomenon is not limited to mercury as revealed in several reports. Co-existence of *OqxAB, blaCTX-M, pco* (copper) and silver (*sil*) resistance operons on the same plasmid have been reported (Mourao et al. 2015; Fang et al. 2016). The co-selection of cadmium and zinc for methicillin resistance in *Staphylococcus aureus* through HGT of plasmids encoding genes for methicillin (*mec*) and Cd/Zn resistance (*czr*) have also been reported (Cavaco et al. 2010). In addition, the mobile colistin resistance gene, *mcr-1* has been found in copper-tolerant isolates (Campos et al. 2016). Furthermore, a Scottish study on randomly selected archived agricultural soils spanning a period of 30 years revealed the presence of 11 antibiotic resistance genes correlated to soil copper, chromium, lead and iron levels (Knapp et al. 2011).

Cross-resistance between antibiotics and heavy metals has also been documented. For instance, efflux systems have been reported to confer cross-resistance to metals and antibiotics (Cheng et al. 1996; Mata et al. 2000). In addition, co-regulation of antibiotic and metal resistance has been reported in *Pseudomonas aeruginosa* cadmium-zinc-cobalt regulatory system, CzcRS, which regulate not only the expression of the *czcCBA* efflux system that confers resistance to cadmium, zinc, and cobalt but also decrease expression of OprD porin, resulting in increased resistance to carbapenems (Perron et al. 2004).

From the foregoing, it is highly plausible that the spread and dissemination of antibiotic resistance genes in 3S metagenome are facilitated by the presence of heavy metal resistance genes, which are often borne on mobile genetic elements that also carries resistance determinant for various antibiotic classes. This assertion is further corroborated by the fact that most of the mobile genetic element-encoded heavy metal resistance genes detected in this study have been reported in the literature to also carry antibiotic resistance genes. Furthermore, exposure to multiple heavy metals, as obtained in this study, has been shown to be more effective for co-selection for antibiotic resistance (Guo et al. 2014). More so, it has been observed that the concentrations of heavy metals in natural environments, which at times can be lower than the minimum inhibitory concentration may be enough to drive the co-selection of antibiotic resistance genes (Seiler and Berendonk 2012), and this has also been demonstrated in in vitro studies (Gullberg et al. 2014).

Conclusions

In summary, this study has unravelled via next-generation shotgun metagenomics diverse antibiotic and heavy metal resistance genes constituting the resistome of 3S metagenome. Antibiotic resistance genes for 15 antibiotic classes and heavy metal resistance genes of various heavy metals were detected, their resistance mechanisms explained and the public and environmental health implications of their presence enunciated. The preponderance in this study, of resistance genes against antibiotics used to treat infections caused by members of the family *Enterobacteriaceae* points to possible contamination from faecal materials of medicated humans and animals via point and non-point sources. Attempt was made to establish the possible contributions of the heavy metal resistance genes to the spread and dissemination of antibiotic resistance determinants in the metagenome. The huge repository of antibiotic and heavy metal resistance genes in the soil environment poses a significant threat to public and environmental health, and due to the intricate link between humans and the soil, it may be a huge contributor to the proliferation of multidrug-resistant clinical pathogens.

Author contributions LBS designed, execute, analyse and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The author declares that there is no conflict of interest.

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