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Genomic analysis of ST117, 155, 1011, 167, 744, and 17391 in poultry-associated multidrug resistant *Escherichia coli* isolates from India

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Multidrug-resistant *Escherichia coli* originating from poultry farms pose a significant One Health threat because of their emergence and spread connecting agricultural farms and the environment causing infections in humans. In this study, 38 isolates were collected, all of which exhibited resistance to penicillin, cephalosporins, fluoroquinolones and tetracycline, with an average Multiple Antibiotic Resistance (MAR) Index of 0.55. Among these 38 isolates, 7 isolates having ≥ 0.6 MAR index were subjected to whole genome sequencing - KEED-2 (ST117), KEED-3 (ST155), MTBW-1 (ST1011), MTBW-2 (ST167), PUND-1 (ST117), PUND-3 (ST17391) and VELW-1 (ST744). The analysis revealed the presence of antimicrobial-resistance genes such as tetracycline *(tet(A))*, quinolone *(QnrS1)* and aminoglycosides (*aph(4)-Ia*, *aac(3)-IVa*, *aac(3)-IId*, *aph(3'')-Ib*, *aph(3')-Ia*, *aph(6)-Id*). Notably, the *CTX-M* gene was present in KEED-2 (ST117), and the *TEM-1B* gene was present in MTBW-1 (ST1011) and VELW-1 (ST744). In this study, pandemic clones ST167, ST744 and ST17391 were identified, which has not been reported so far in poultry environments in India to the best of our knowledge, highlighting the need for continued surveillance and effective control measures, emphasising significance for the One Health framework.

Keywords Poultry bedding material, Water, MDR *Escherichia coli*, Whole genome sequencing

Antimicrobial drugs are used worldwide to maintain livestock's health and productivity[1]. Multidrug resistant *Escherichia coli* strains are increasingly prevalent in poultry environments, posing substantial public health risks[2]. Food animals and their production environments serve as notable reservoirs of resistant bacteria and resistance genes. The transmission of these elements to humans can occur through direct contact, food production chain, or via the dissemination of animal waste onto land[3]. Animals excrete a substantial proportion of antibiotic doses, with up to 75% found in faeces and 90% in urine[4]. Various factors contribute to the proliferation of resistance at the farm level, including large animal populations in confined spaces, substandard sanitation, and the uncontrolled application of broad-spectrum antimicrobials[5]. Antimicrobial-resistant *Escherichia coli* originating from poultry may disseminate across environments and may present as a risk to both human health and the poultry industry[6]. India's poultry industry is rapidly expanding, currently holding the seventh position worldwide in meat production. To meet the escalating demand for poultry meat in India, poultry farming has seen significant growth, especially in key poultry-producing states such as Tamil Nadu and Andhra Pradesh. Global chicken imports rose by 4% annually between 2001 and 2021, reaching 14.2 million metric tonnes, making chicken the world's most consumed livestock item. The USDA forecasts a further increase to 17.5 million metric tonnes by 2031 (https://ers.usda.gov/). In Gujarat, ST681 was predominantly present in ESBL-producing *E. coli* isolates and found to be associated with virulence factor and plasmid-mediated antimicrobial-resistant genes[7]. In Punjab (India), the ESBL genes such as *blaCTX-M* and *TEM* have been found to be the predominant resistance determinants that confer resistance to beta lactam antibiotics used in poultry farms and veterinary

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medicines[8]. Previous studies from India have reported the presence of ESBL clones such as ST131, ST10, and ST117 of *Escherichia coli* from broiler from different states in Telangana, Andhra Pradesh, and Karnataka[9]. The ST167 strain of *E. coli* is common in India and is strongly associated with multidrug resistance, being identified as the predominant sequence type among carbapenem-resistant *E. coli* isolates in the country[10]. Although there has been no reports of ST744 in India so far, our study reveals the presence of ST744 and also, multiple studies from China, Japan and Pakistan have reported the presence of ST744 carrying beta-lactamases and colistin resistance gene (*mcr1.1*) from clinical and poultry associated environments[11]. Continuous genomic surveillance is being conducted in developed countries such as the United Kingdom, Netherlands and Germany. But India lacks sufficient data in the sequencing aspect and has only 29 WGS data from poultry till date. Hence, we investigated the antibiotic resistance pattern of the bedding material and water to study the prevalence of multidrug-resistant *Escherichia coli* strains in the poultry environment and subsequently identify the Sequence types circulating in the poultry farms.

Materials and methods

Study area and sample

A total of 38 samples (bedding material and water) were collected from 7 different poultry farms in Tamil Nadu. These samples were packed in sterile falcon tubes and processed within 24 h.

Isolation of *Escherichia coli*

0.5 g of each sample (bedding material) was inoculated into 5 ml of BHI broth and 1 ml of water samples were inoculated into MacConkey broth (HiMedia). All samples were incubated at 37 °C for 24 h. Subsequently, samples were spread on Eosin Methylene Blue (EMB) agar plates (HiMedia) and incubated at 37 °C. The presence of a green metallic sheen confirmed the *Escherichia coli.* The isolates were confirmed by MALDI-TOF analysis before sequencing.

Antimicrobial susceptibility testing

A total of 13 antibiotic discs (Himedia) belonging of 10 classes of drugs were used to determine the resistance pattern of *Escherichia coli* isolates: ampicillin (10 mcg), cephalexin (30 mcg), ceftazidime (30 mcg), ofloxacin (5 mcg), tetracycline (10 mcg), chloramphenicol (10 mcg), co-trimaxozole (10 mcg), gentamicin (10 mcg), nitrofurantoin (300 mcg), ertapenem (10 mcg), colistin (10 mcg), amoxicillin-clavulanate (10 mcg). The Antimicrobial Susceptibility tests were carried out using CLSI 2023 guidelines. ATCC 25922 was used as a control strain.

Whole genome sequencing

The genomic DNA was isolated using the DNeasy Ultraclean Microbial Kit and the library was prepared using the KAPA Hyperplus Kit. The Novaseq 6000 from Illumina was used to sequence entire genomes. The Phred Quality Score (Q Score), with a cutoff value of > 30, was used to screen the raw data. The Illumina adapter sequences were extracted from both matched end-data fastq files. Adapter removal and de novo assembly were done using Trim Galore v0.6.10 and Unicycler assembler v0.4.8, respectively[12].

Shotgun metagenomic analysis

Total DNA was extracted from the poultry bedding material and the DNA was quantified using the Qubit Fluorometer. Sequencing libraries were constructed and quantified. Libraries were sequenced on an Illumina NovaSeq 6000 platform, generating paired-end reads. Raw data underwent quality trimming before bioinformatics analysis. The Kraken taxonomic composition of the sample was determined, and the Antimicrobial Resistance Genes (ARGs) were identified and quantified using ABRicate v1.0.1.

Data analysis

Multi-locus sequence typing (MLST) v2.0 was used to determine the strain's taxonomic position, and the ABRicate v1.0.1 was used to determine the antibiotic-resistant genes. VirulenceFinder v2.0 and PlasmidFinder v2.0 were used to identify the virulence genes and plasmid replicons. The pangenome analysis was carried out using Roary pipeline 3.13.0 was used to detect the shell genes, core genes and accessory genes using annotation files generated using Prokka 1.14.6. The output files from the Roary pipeline were used for pangenome analysis using Phandango v1.3.1.

RESULTS

Phenotypic analysis

A total of 38 samples were collected from bedding material and water across different farms. Among these, KEED-2, KEED-3, MTBW-1, MTBW-2, PUND-1, PUND-3 and VELW-1 isolates tested positive for *Escherichia coli*. The Kirby-Bauer assay was used to determine the samples resistance to antibiotics such as ofloxacin 91.3% (21/23), tetracycline 86.9% (20/23), ampicillin 82.6% (19/23) and amoxicillin-clavulanate 69.5% (16/23) (Table 1). Average MAR of 0.55 indicates the excessive use of antibiotics in poultry. 7 samples having MAR index of > 0.6 were subjected to whole genome sequencing.

Genotypic analysis

Bedding material

The two *Escherichia coli* isolates (KEED-2 & PUND-1) from bedding material are phenotypically resistant to penicillin, cephalosporin, fluoroquinolone, and tetracycline. Whole genome analysis revealed both isolates

Antibiotics ($n=13$)	Total samples tested ($n=23$)	Total resistant isolates	% Resistant
Ampicillin (AMP)	23	19	82.6%
Cefalaxin (CN)	23	14	60.8%
Ceftazidime (CAZ)	23	13	56.5%
Cefixime (CFM)	23	12	52.1%
Ofloxacin (OF)	23	21	91.3%
Tetracycline (TET)	23	20	86.9%
Chloremphenicol (C)	23	9	39.1%
Co-trimaxazole (COT)	23	11	47.8%
Gentamicin (GEN)	23	6	26.0%
Nitrofurantoin (NIT)	23	6	26.0%
Ertapenem (ETP)	23	0	0%
Colistin (CL)	23	15	65.2%
Amoxicillin-clavulanate (AMC)	23	16	69.5%

Table 1. Antibiotic resistance in *Escherichia coli* isolates.

Sample ID	Sequence type (MLST)	AMR genes	Virulence genes	Plasmids
KEED-2	ST117	*blaCTX-M-15*, *tet(A)*, *qnrS1*,*aph(3')-Ia*, *mdf(A)*	*yehB*, *traT*, *sitA*, *ompT*, *iutA*, *iucC*, *irp2*, *hlyF*, *fyuA*, *fimH*, *mchF*	IncFIB, IncFIC, IncI-gamma/K1, IncX4
KEED-3	ST155	*mdf(A)*	*fimH*, *ipfA*, *terC.hlyE*	-
PUND-1	ST117	*tet(A)*, *qnrS1*, *mdf(A)*	*chuA*, *cvaC*, *etsC*, *fyuA*, *hlyE*, *iss*, *iutA*, *iroN*, *iucC*, *ompT*, *sitA*.	IncFIB, IncFIC, IncI-gamma/K1
PUND-3	ST17391	*tet(A)*, *mdf(A)*, *qnrS1*, *sul3*, *aph(3')-Ia*, *ant(3")-Ia*	*csgA*, *fimH*, *papC*, *sfa*	IncX1, IncY
MTBW-1	ST1011	*mdf(A)*, *tet(A)*, *aac(3)-IVa*, *aph(4)-Ia*, *aadA*, *ant(3")-Ia*, *cmlA1*, *catA1*, *sul3*, *blaTEM-1B*	*Tia*, *fimH*, *chuA*, *astA*	Col(MG828)
MTBW-2	ST167	*mdf(A)*, *blaTEM-1B*	*Cma*, *csgA*, *cvaC*, *fimH*, *hlyE*, *hlyF*, *iroN*, *iss*, *iucC*, *iutA*, *ompT*, *sitA*, *terC*, *traT*.	IncFIB, IncFIC
VELW-1	ST744	*mdf(A)*, *mph(A)*, *sul1*, *sul2*, *sul3*, *tet(A)*, *tet(B)*, *aadA5*, *dfrA1*, *ant(3")-Ia*, *catA1*, *aph(3")-Ib*, *aph(6)-Id*, *aac(3)-IId*, *aph(3')-Ia*, *blaTEM-1B*	*Cma*, *csgA*, *cvaC*, *fimH*, *hlyF*, *hlyE*, *iss*, *sitA*.	IncX1, IncX3, IncX4, IncFIB, IncQ1

Table 2. Genomic analysis of *Escherichia coli* isolates.

belong to ST117 from different poultry bedding materials. The isolate (KEED-2) harboured several antimicrobial resistance genes, including *aph(3')-la*, which confers resistance to aminoglycoside, *blaCTX-M-15*, encoding an extended spectrum beta-lactamase, *mdf(A)*, associated with multidrug efflux, *QnrS1*, mediating resistance to fluoroquinolones, and *tet(A)* responsible for tetracycline resistance (Table 2). The isolate (PUND-1) harbours multi drug efflux pump *(mdfA)*, tetracycline resistant gene *(tet(A))* and fluoroquinolone-resistant gene *QnrS1* (Table 2). The ST117 of both isolates (KEED-2 & PUND-1) contains different types of plasmids (IncFIB, IncFIC, IncQ1, IncX4) as identified by PlasmidFinder v2.0. The isolate (PUND-3) was found to be phenotypically resistant to 9 out of 10 classes of drugs, such as penicillin, cephalosporin, tetracycline, ofloxacin, phenicol, sulfamethoxazole, aminoglycoside, nitrofurantoin and colistin. Whole genome sequencing of the isolated PUND-3 revealed a new sequence type ST17391. The PUND-3 carries antimicrobial-resistant genes such as *tet(A)* (tetracycline), *qnrS1* (quinolone), *sul3* (sulafamethoxazole) and *aph(3')Ia-3*, *ant(3")Ia-1* (aminoglycoside). Also this isolate carries IncY (79255 bp) and IncX1 (43410 bp) plasmids. The isolate (KEED-3) was found to be resistant to penicillin, cephalosporin, fluoroquinolone, tetracycline, phenicol, sulfamethoxazole and aminoglycosides. The multidrug resistant *E.coli* isolate (KEED-3) belongs to the ST155, and it carries multidrug efflux pump *mdf(A)* gene.

Water

The isolate VELW-1 was found to be resistant to penicillin, fluoroquinolone, tetracycline, sulfamethoxazole and aminoglycosides. The multidrug-resistant *E. coli* (VELW-1) belongs to ST744. This isolate carries multiple resistance genes, such as *aadA1*, *aadA5*, *aac(3)-IId*, *aph(3')-Ia*, *aph(3")-Ib*, *aph(6)-Id* (aminoglycoside), *sul1*, *sul2*, *sul3* (sulfamethoxazole), *blaTEM* (beta lactamase), *catA1* (phenicol), including multiple plasmids types (IncFIB, IncX1, X3, X4, IncQ1) (Table 2). The isolate MTBW-2 belongs to ST167. It carries the *TEM-1B* beta lactamase and *mdfA* gene, conferring resistance to multiple antibiotics. The isolate MTBW-1 belong to ST1011 and harbours resistance genes such as *TEM-1B* (beta-lactamase), *aac(3)-IVa*, *aph(4)-Ia*, and *aadA3* (aminoglycoside), *sul3* (sulfamethoxazole) and *catA1* (phenicol) genes respectively and also carrying colMG828 plasmid (Table 2).

Virulence genes

The multidrug-resistant isolates harbours several important virulence genes. Frequently detected virulence factors include adhesins (fimbriae), iron acquisition systems (*iroN*, *iutA*, *iucC*, *sitA*) and protectins (*iss*) (Table 2). Specifically, *iss* was identified in 6 out of 7 isolates pointing to serum-resistant capabilities. The *IroN* gene encoding for catecholate siderophore receptor and *iutA/iutC* genes involved in the aerobactin-mediated iron uptake were present in all isolates respectively, indicating strong iron acquisition potential[13]. The detection of *papC* in these isolates (PUND-3) indicates this strains may have the potential to colonise in the urogenital tract of humans or animals[14]. The co-occurrence of *papC* and *sfa* genes from non-clinical sources such as poultry bedding material raise concern about the silent circulation of virulent *E. coli* strains in livestock settings[15].

Shotgun metagenomic analysis of MTB-3

Whole genome sequencing of *E. coli* isolates MTBW-1 and MTBW-2, obtained from water samples, identified ST1011 and ST167 respectively. Both isolates exhibited resistance to tetracycline, chloramphenicol, beta-lactamase, and aminoglycosides and contained key mobile genetic elements linked to their spread. Given its pandemic potential (ST167) and to understand the broader resistome within the farm environment, metagenomics was subsequently carried out using poultry bedding material from the same farm.

The metagenomic analysis of the bedding material (MTB-3) reveals the microbial ecosystem being reservoirs of several drug-resistant genes, including tetracycline, phenicol, lincosamide, fluoroquinolone, penicillin, macrolide, sulfonamide and phosphonic acid in this isolate (Table 3). The taxonomic analysis of MTB-3 revealed the predominance of bacteria (≅74%) (Fig. 5). The tetracycline, phenicol and sulfonamide were the most resistant genes across MTBW-1, MTBW-2 and MTB-3 samples (Fig. 6). These genes represent the farm's core resistome and selective pressures may be due to the usage of antibiotics in poultry.

Discussion

The present study reveals a high prevalence of multidrug-resistance among *E. coli* isolated from poultry bedding material and water samples. Whole genome sequencing revealed several internationally recognised high-risk sequence types (STs), including ST117, ST167 and ST744, which have been frequently linked to avian pathogenic *E. coli*[16]. ST117 isolates carry clinically relevant genes such as *blaCTX-M-15*, *QnrS1* and *tet(A)* and it is known to act as a reservoir for resistance determinants against important antibiotics. Poultry, especially ST117 lineage, is known to be the likely source of zoonotic strains[17]. ST744 isolate was found to carry *tet(A)*, *tet(B)*, *sul1*, *sul2*, *sul3*, *mdf(A)*, *catA1* and *blaTEM-1B*. Globally there have been reports of ST744 harbouring resistance genes such as *tet(A)*, *mdf(A)*, *catA*[18]. Additionally, mcr-1 positive isolates from poultry in Romania[19] and swine samples in China have been reported to carry ST744[20]. A recent report from Indonesia stated that the ST744, carrying multiple AMR genes, including *blaCTX-M-15*, was reported in hospital wastewater[21]. Phylogenetic analysis of ST744 was carried out along with the poultry isolates across the globe (Figs. 1 and 2). The presence of core genes 3753 (23%), soft core genes 175 (1.1%), shell genes 1424 (8.7%) and cloud genes 2818 (17.2%), with a total of 8,170 (50.0%) genes identified across all strains. The phandango analysis illustrates the distribution of selected antimicrobial resistance genes (*mdfA*, *mcr1.1*, *catA1*, *tet(A)*) and virulence genes (*papC*, *pitA*, *iutA*, *iutB*, *hlyE*, *iucA*, *iucC*, *iucD*) in the isolates. ST167 is a recognised high-risk extraintestinal pathogenic *E. coli* clone predominantly associated with multidrug resistance and carbapenem resistance, particularly *NDM* variants posing significant therapeutic challenges. Phylogenetic analysis of ST167 *E. coli* isolates from this study with 50 previously reported ST167 isolates from different sources in India was provided (Figs. 3 and 4). Pangenome analysis (Fig. 3) using Roary to identify a total of 10,395 (50%) gene clusters. Among these, 3,617 (17.4%) were core genes present in nearly all strains, 272 (1.3%) soft core genes, 1240 (6.0%) shell genes and 5,266 (25.3%) cloud genes. The phandango analysis (Fig. 4) focused on key antimicrobial resistance genes *(mdfA*, *blaNDM-1*, *blaTEM-1B*, *emrA*, *emrB*) and virulence factors (*ompT*, *iutA*, *iucC*, *iss*, *iroN*) in *E. coli* ST167 isolates, demonstrating their distribution patterns and genetic relationships. Recent studies reveal that ST167 isolates in India harbour diverse resistance and virulence determinants, including *blaNDM* and *blaCMY* genes, posing significant challenges to public health[22]. ST167 has been reported in India from clinical and environmental sources, with one or two reports from livestock. However, there are currently no reports of ST167 from poultry environments in India based on the data from Enterobase v.1.2.0. ST167 has been identified in this study, and

Drug class	Antibiotics	Genes
Tetracycline	Tetracycline	*tet(M)*, *tet(L)*, *tet(K)*, *tet(W)*, *tet(X)*, *tet(Z)*, *tet(A)*, *tet(33)*, *tet(39)*
Macrolide	Azithromycin Erythromycin	*msr(D)* *mph(E)*, *mph(C)*, *mef(A)*, *msr(D)*
Penicillin	Amoxillin-clavulanic acid	*mecA*
sulfonamides	Trimethoprim, sulfamethoxazole	*dfrA1*, *dfrA15*, *dfrA16*, *dfrA17*, *dfrG*, *dfrD.* *sul1*, *sul2.*
Linosamide	Lincomycin, Clindamycin	*erm(X)*, *erm(C)*, *erm(A)*, *erm(T)*, *erm(F)*, *erm(G)*, *erm(Y)*, *Inu(A)*, *Inu(B)*, *Inu(C)*, *Inu(D)*, *Inu(G)*, *Inu(F).* *vga(E)*
Phenicol	Chloramphenicol	*Cmx*, *cat(pC233)*
Fluroquinolone	Ciprofloxacin	*qnrD1*
Phosphonic acid	Fosfomycin	*fosD*

Table 3. Shotgun metagenomics analysis-antimicrobial resistance genes present in MTB-3.

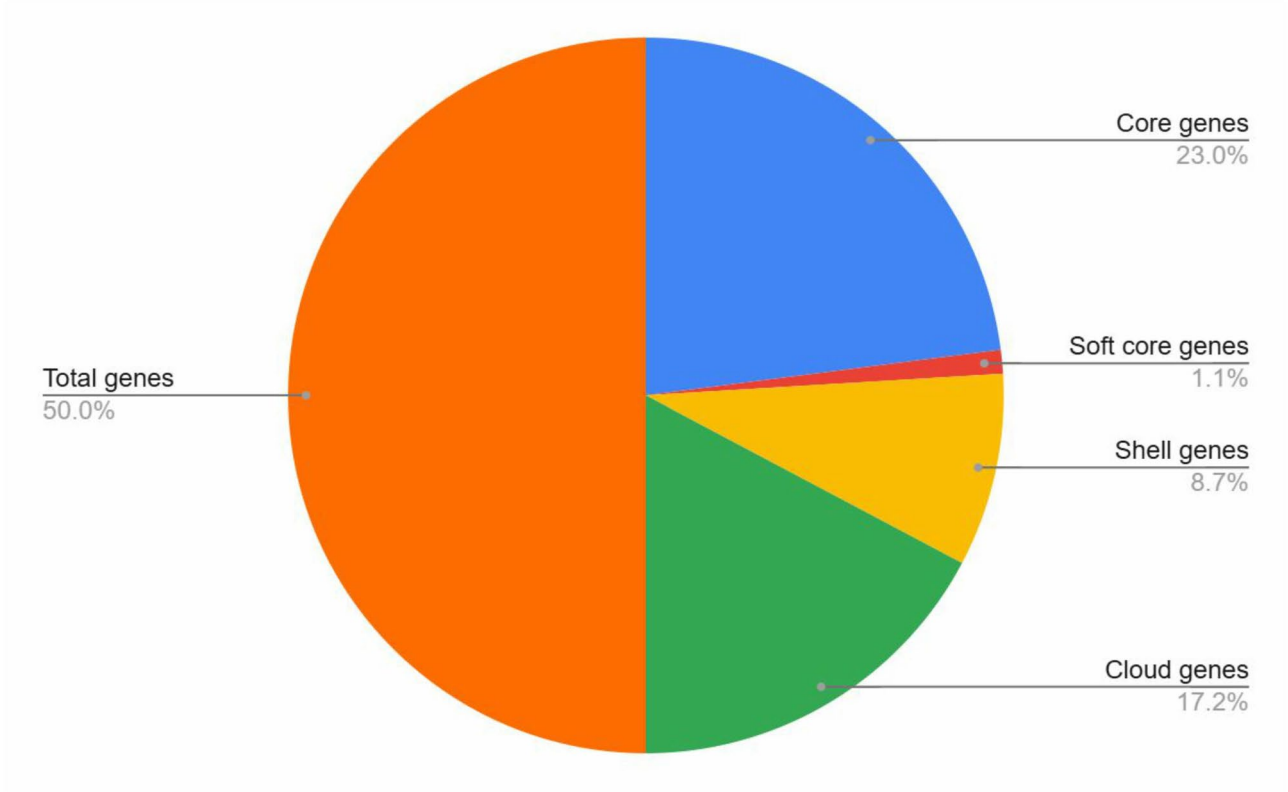

Fig. 1. Pan Genome analysis ST744 with the presence of different types of genes such as Core genes, Softcore genes, Cloud genes and Shell genes.

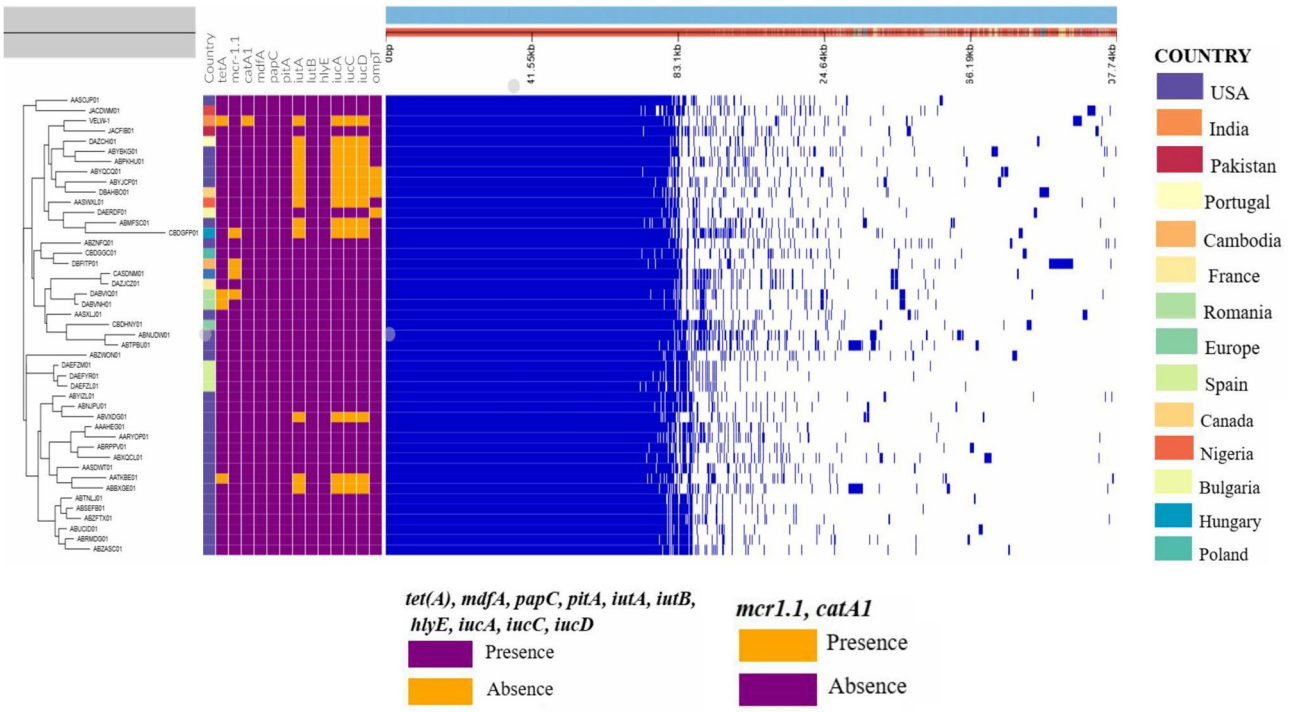

Fig. 2. Illustration of the presence of various different antimicrobial and virulence genes in Asian poultry isolates.

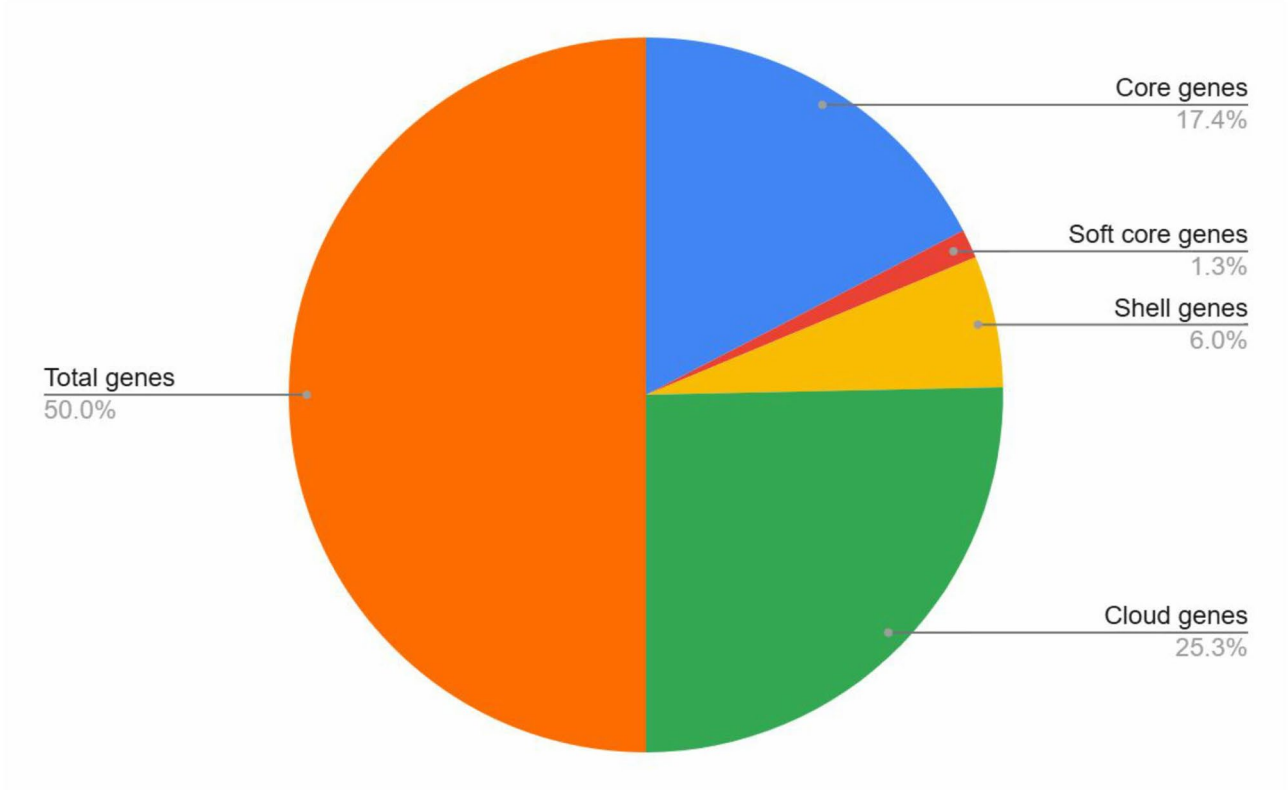

Fig. 3. Pan Genome analysis of ST167 with the presence of different types of genes such as core genes, softcore genes, cloud genes and shell genes.

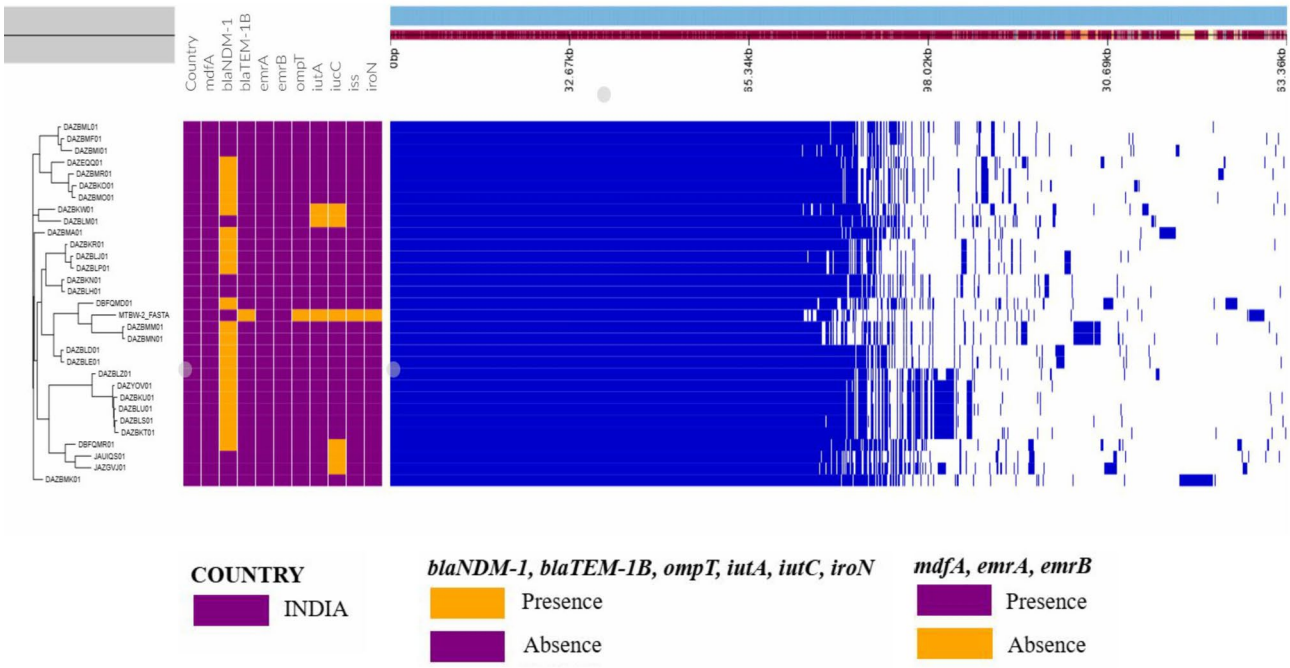

Fig. 4. Illustration of the presence of various different AMR and virulence genes in Indian isolates.

this is the first report of ST167 from poultry environments in India to the best of our knowledge. Our study also reported a new sequence type 17391 from poultry bedding material carrying *qnrS1*, *tet(A)* and *sul3* genes which has not been reported globally so far. The emergence of a new sequence type from poultry bedding material is of particular concern as it highlights the role of AMR within poultry environments and underscores the importance of genomic surveillance[23]. When compared to other countries, India exhibits a low number of sequencing reports. Hence, enhanced WGS, and increased metagenomic surveillance could facilitate better control measures (Figs. 5 and 6).

Conclusion

These findings collectively highlight the critical need for a One Health approach to effectively understand and control antimicrobial resistance in poultry environments. Nowadays, the agricultural farms are getting converted to poultry farms because of the regular income provided by the companies. Additionally, the bedding material is disposed of into agricultural lands, and the residual antibiotics or the resistant bacteria present in it are released into the environment (soil, water) via poultry litter. Routine use of antibiotics in poultry farming increases the risk of antibiotic-resistant bacteria emerging, which can transfer to humans. This transfer threatens public health by complicating the treatment of human infections and undermining efforts toward universal health coverage and control of communicable diseases (SDG 3). As advocated by the government (https://cpcb.nic.in/), the disposal protocols have to be routinely implemented in coordination with the poultry farms and the respective animal husbandry department. Adhering to proper waste disposal and monitoring guidelines in poultry farms is essential for effective poultry management. Continuous surveillance of the poultry farms should be performed using whole genome sequencing to know the different ST types circulating in the poultry farms along with the

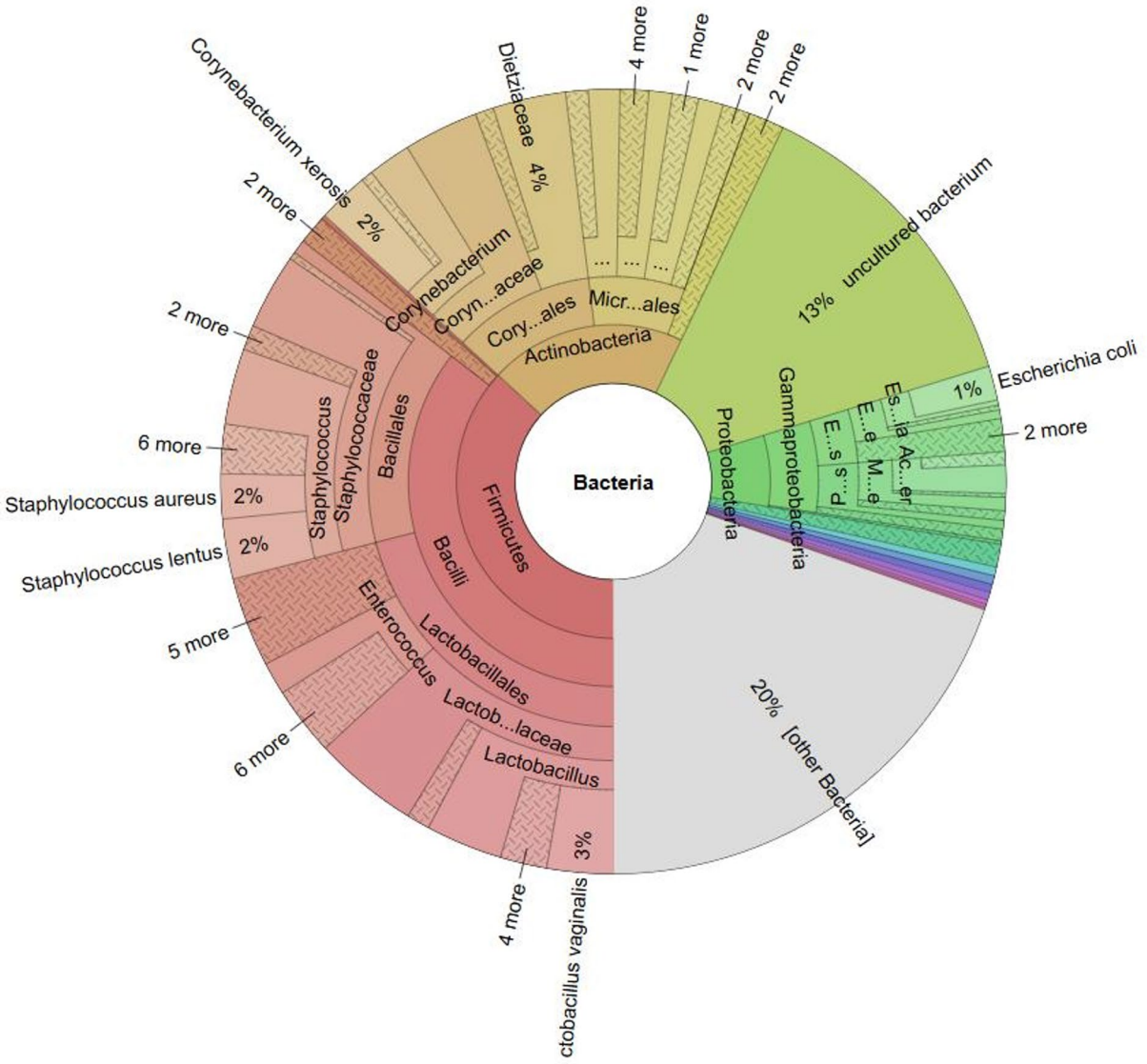

Fig. 5. Krona pie chart MTB-3.

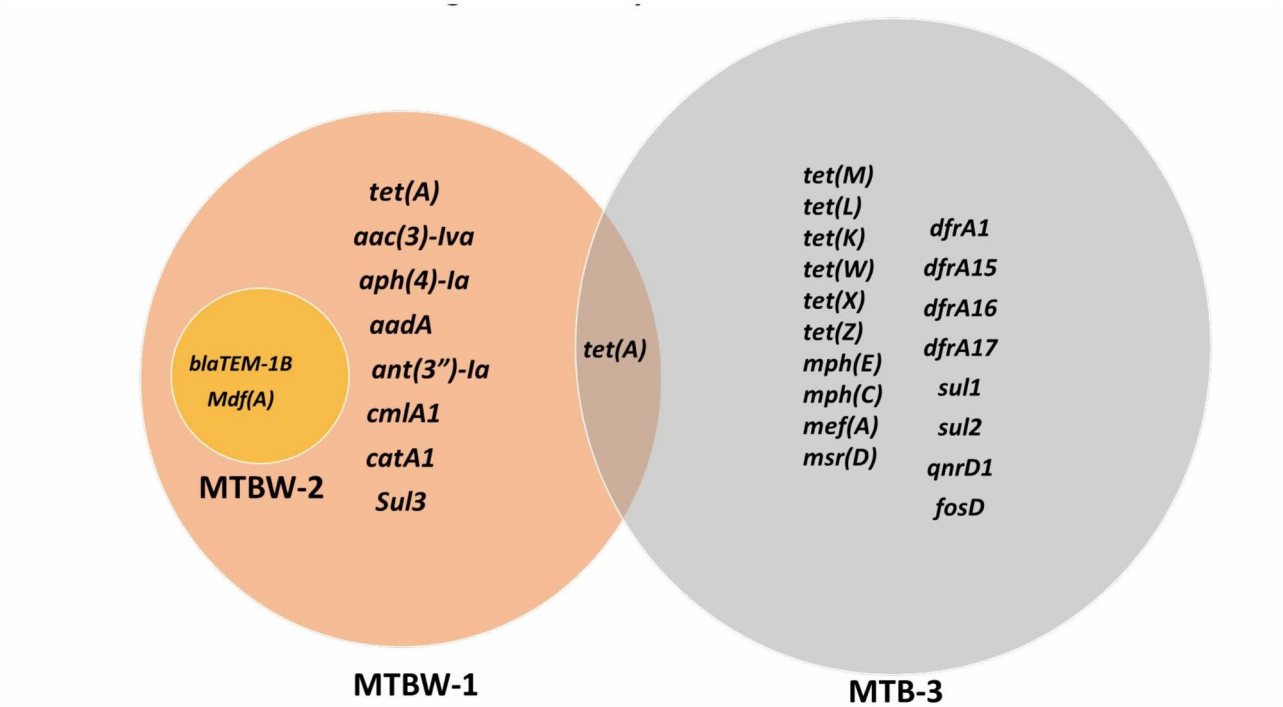

Fig. 6. Venn diagram representing the correlation between the whole genome sequence and metagenome from MTB farm.

shotgun metagenomics approach to provide the data needed to advocate for responsible management practices in the farms (SDG 12).

Data availability

The sequence data for the samples has been submitted in the NCBI. The Sample ID KEED-2 has the Nucleotide accession IDJBEXCF000000000.1, Bioproject ID PRJNA224116, and Biosample ID SAMN42315190. KEED-3 corresponds to Nucleotideaccession ID JBEXCG000000000.1, Bioproject ID PRJNA1131611, and Biosample ID SAMN42315416. PUND-1 has the Nucleotideaccession ID JBRJJE000000000.1, Bioproject ID PRJNA1333720, and Biosample ID SAMN51805429. For PUND-3, the Nucleotideaccession ID is JBLOHC000000000.1, Bioproject ID is PRJNA1201741, and Biosample ID is SAMN45941455. The MTBW-1 samplehas the Nucleotide accession ID JBRZAD000000000.1, Bioproject ID PRJNA1355167, and Biosample ID SAMN53032474. MTBW-2is associated with Nucleotide accession ID JBRJJF000000000.1, Bioproject ID PRJNA1333795, and Biosample ID SAMN51805480.Finally, VELW-1 has the Nucleotide accession ID JBRJJG000000000.1, Bioproject ID PRJNA1333918, and Biosample IDSAMN51819988. MTB-3 (Metagenome sample) has the Bioproject ID PRJNA1347992.

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Declarations

Competing interests
The authors declare no competing interests.

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