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Whole genome sequencing analysis on antibiotic-resistant *Escherichia coli* isolated from pig farms in Banten Province, Indonesia

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ABSTRACT

Importance: The emergence and rapid increase in the incidence of multidrug-resistant (MDR) bacteria in pig farms has become a serious concern and reduced the choice of effective antibiotics.
Objective: This study analyzed the phylogenetics and diversity of antibiotic resistance genes (ARGs) and molecularly identified the source of ARGs in antibiotic-resistant *Escherichia coli* isolated from pig farms in Banten Province, Indonesia.
Methods: Forty-four antibiotic-resistant *E. coli* isolates from fecal samples from 44 pig farms in Banten Province, Indonesia, were used as samples. The samples were categorized into 14 clusters. Sequencing was performed using the Oxford Nanopore Technologies MinION platform, with barcoding before sequencing with Nanopore Rapid sequencing gDNA-barcoding (SQK-RBK110.96) according to manufacturing procedures. ARG detection was conducted using ResFinder, and the plasmid replicon was determined using PlasmidFinder.
Results: Three phylogenetic leaves of *E. coli* were identified in the pig farming cluster in Banten Province. The *E. coli* isolates exhibited potential resistance to nine classes of antibiotics. Fifty-one ARGs were identified across all isolates, with each cluster carrying a minimum of 10 ARGs. The *ant(3′′)-Ia* and *qnrS1* genes were present in all isolates. ARGs in the *E. coli* pig farming cluster originated mainly from plasmids, accounting for an average of 89.4%.
Conclusions and Relevance: The elevated potential for MDR events, coupled with the dominance of ARGs originating from plasmids, increases the risk of ARG spread among bacterial populations in animals, humans, and the environment.

Keywords: Antibiotic resistance; antibiotic resistance genes; *Escherichia coli*; multidrug-resistant; pig farm

INTRODUCTION

Antimicrobial resistance (AMR) is a critical and imminent threat to global public health. The swift rise of multidrug-resistant (MDR) pathogens, particularly those resilient to last-

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Conflict of Interest
The authors declare no conflicts of interest.

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resort antibiotics, such as carbapenems, colistin, and tigecycline, raises serious concerns, narrowing the spectrum of effective antibiotic choices [1,2].

The extensive use of antimicrobials in livestock contributes significantly to the escalation of AMR on a global scale. Approximately 73% of all antimicrobials sold worldwide are deployed in livestock for growth promotion (Antibiotic Growth Promoter), increased production, and disease prevention [3]. In particular, a previous study indicated the highest prevalence of antibiotic-containing feed in pigs (55.4%) compared to chickens (42.2%), quail (18.9%), and ducks (9.2%) [4]. This widespread and unregulated use exerts selective pressure on bacteria, fostering the proliferation of resistant strains capable of spreading across human, animal, and environmental bacterial populations [5].

Certain antibiotic-resistant bacteria, particularly *Escherichia coli* [6], are of global concern. *E. coli*, a Gram-negative bacterium in the Enterobacteriaceae group, is a primary cause of foodborne infections and serves as a key reservoir for antibiotic resistance genes (ARGs). The propensity of *E. coli* for accumulating ARGs, predominantly through horizontal gene transfer facilitated by various mobile genetic elements (MGEs), particularly plasmids, underscores its essential role in disseminating multidrug resistance genes between diverse bacterial species [7]. Numerous studies highlight the role of *E. coli* in propagating critical ARGs, such as *blaNDM-1*, *mcr*, and *tet(X3)/(X4)*, limiting the efficacy of antibiotics, including last-resort options (carbapenems, colistin, and tigecycline) and progressively diminishing the pool of effective treatments for human and animal health [1,2]. Furthermore, the emergence of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* compounds poses a global health challenge [5], making *E. coli* a vital biomarker for monitoring AMR in livestock on farms and in hospitals [7].

The advent of whole genome sequencing (WGS) represents a groundbreaking advancement in molecular technology, offering extensive, high-resolution insights into pathogen subtypes. Leveraging WGS for the monitoring and surveillance of AMR is imperative because it provides critical information on the initial emergence and spread of resistance. The data highlight the need to develop effective policies to combat AMR. Sequencing data from AMR surveillance is integral for developing rapid diagnostic tools, complementing traditional phenotypic testing methods [8].

Oxford Nanopore Technologies (ONT) MinION is one of the platforms used for WGS on bacteria. The advantage of the ONT MinION platform is that its third-generation sequencing technology can generate long reads (up to 2.27 Mbp) that can span most repetitive sequences and provide the opportunity to link ARGs and their flanking regions, accurately identifying populations carrying ARGs. The ONT MinION platform can generate raw data in real-time, making it more accessible and efficient for genome assembly and complex structural detection [9]. ONT MinION produces long DNA/RNA sequences and is a portable, pocket-sized sequencing device that does not require PCR or chemical labeling during preparation [10].

Indonesia faces challenges in antibiotic use in the livestock industry, particularly in pig farming, coupled with inadequate waste treatment systems, posing a significant risk of spreading resistant bacteria to the broader environment and a severe threat to public health. Moreover, there is no comprehensive data on antimicrobial-resistant *E. coli* genotypes in pig farms, particularly in Banten Province. This study characterized the phylogenetic and ARG diversity and the molecular features of ARG-containing plasmids in antibiotic-resistant *E. coli* from pig farms in Banten Province. Research on molecular antibiotic resistance is crucial for predicting the resistance rates and is a foundational basis for controlling antibiotic resistance.

METHODS

Animal care

This research was exempt from ethical approval and Institutional Animal Care and Use Committee (IACUC) clearance because it did not involve the treatment of animals. Nevertheless, sample collection adhered to established protocols outlined in the Global Tricycle Surveillance ESBL *E. coli* from WHO, 2021 [11].

Sample collection and preparation

Forty-four antibiotic-resistant *E. coli* isolates obtained from fecal samples at 44 distinct pig farms in Banten Province, Indonesia, were used as samples. The samples were isolated and identified in a prior study [12,13]. These *E. coli* isolates were categorized into 14 clusters (pooling), determined by shared characteristics and proximity to the respective farm areas. *E. coli* was isolated and identified according to the protocols outlined in the Global Tricycle Surveillance ESBL *E. coli* from WHO, 2021 [11]. Antibiotic susceptibility testing of *E. coli* isolates used the Kirby–Bauer disk diffusion method on MHA media, referring to the Clinical and Laboratory Standards Institute (CLSI) 2018 [14].

DNA extraction and DNA quality control

E. coli DNA extraction was performed using a PowerWater DNA extraction kit (Qiagen, Germany) according to the manufacturer's specified procedures. The concentration of extracted DNA was then assessed using a Qubit Fluorometer (Thermo Fisher Scientific, USA).

WGS

The purified DNA was prepared for sequencing on the Oxford Nanopore Technologies (ONT) MinION platform. Before sequencing, it was barcoded using a Nanopore Rapid sequencing gDNA-barcoding kit (SQK-RBK110.96) according to the manufacturer's protocol.

Bioinformatic analysis

The quality of MinION fastq reads was assessed using FastQC. FastQC is a popular and straightforward tool for quality control checks on raw sequence data from high-throughput sequencing pipelines. The tool provides a modular set of analyses that can provide a quick impression of whether data has any problems before further analysis [15]. A mean read length and read quality greater than 1,000 and exceeding 8.0, respectively, met the criteria for successful QC [16]. The initial real-time analysis was conducted through the Oxford Nanopore EPI2ME web tool, followed by further analysis on Galaxy Europe, an online platform for constructing bioinformatic pipelines without command-line dependencies [17]. The ABRicate tool in Galaxy, which integrates data from the CARD database, ResFinder, NCBI, and PlasmidFinder, was used for ARG detection [18]. ARGs were identified using ResFinder, wherein assembled contigs were screened for various classes of antibiotics in the database. ResFinder is an open online resource for identifying ARGs in next-generation sequencing data and predicting the phenotypes from genotypes [19]. Plasmid replicons were determined using PlasmidFinder, a web tool for detecting and characterizing plasmid sequences in WGS data from Enterobacteriaceae [20]. Bioinformatics analysis was used to determine the proportions of multiple ARGs in bacterial samples. The phylogeny was constructed using RAxML (Randomized Axelerated Maximum Likelihood). RAxML is a fast, scalable, and user-friendly tool for maximum likelihood phylogenetic inference [21]. The final phylogenetic tree and heat map were generated using iTOL (Interactive Tree of Life). iTOL is an online tool for displaying, manipulating, and annotating phylogenetics and other trees [22].

Table 1. Quality control of *E. coli* sequencing from fecal samples in pig farm clusters in Banten Province

Farm cluster	Quality control			
	Mean read length	Number of reads	Total bases	Mean read quality
1	2,950	34,207	100,919.39	9.0
2	3,519	20,244	71,252.89	8.5
3	1,839	34,039	62,629.45	9.3
4	4,502	35,847	161,405.93	8.8
5	4,319	57,418	248,034.24	10.2
6	4,398	32,598	143,384.19	10.1
7	4,371	52,101	227,738.50	10.0
8	2,118	53,950	114,308.53	8.7
9	3,147	48,080	151,307.77	9.7
10	2,082	39,805	82,900.50	8.7
11	3,217	57,632	185,449.88	9.8
12	2,083	27,559	57,415.88	8.7
13	3,649	51,634	188,451.33	9.3
14	2,299	35,354	81,311.64	8.8

RESULTS

Quality control of the sequencing results

Sequencing with ONT MinION yielded favorable results for further analysis. The average sequence length ranged from 1,839 to 4,502, with reads numbering from 20,244 to 57,632 and total bases spanning 57,415,877 to 248,034,237. The mean read quality consistently surpassed 8.0, ranging from 8.5 to 10.2 (**Table 1**). These quality control metrics met the requirements, indicating a robust dataset and a reliable foundation for subsequent in-depth analysis.

Phylogenetic analysis and potential multidrug resistance

The results of the phylogenetic analysis conducted on *E. coli* within the pig farming cluster in Banten Province revealed the presence of three distinct phylogenetic branches. In particular, branches 1, 2, and 3 are associated with farm clusters 1, livestock cluster 4, and livestock clusters 6, 2, 5, 11, 7, 14, 3, 10, 8, 13, 12, and 9, respectively. Livestock clusters 8 and 13 exhibited a close genetic relationship, as did clusters 9 and 12, along with clusters 3 and 14 (**Fig. 1**).

E. coli strains derived from fecal samples within these pig farming clusters exhibit a concerning potential for multidrug resistance. In particular, *E. coli* strains from fecal samples in Banten Province's pig farming clusters showed the potential for resistance to

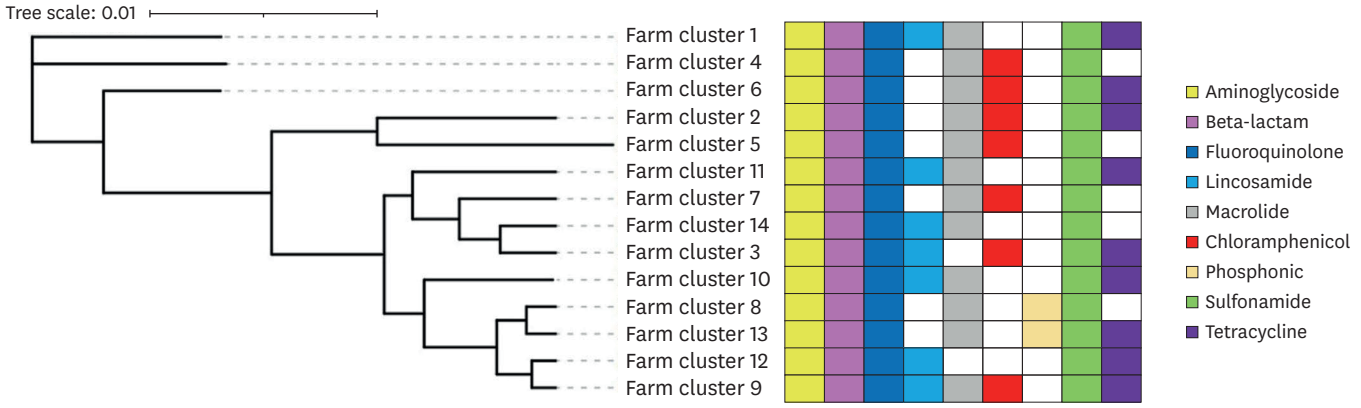

Fig. 1. Phylogenetic tree and multidrug-resistant potential in *E. coli* pig farming clusters in Banten Province.

nine antibiotic classes: aminoglycosides, beta-lactams, fluoroquinolones, lincosamides, macrolides, chloramphenicol, sulfonamides, tetracyclines, and phosphonics. Each livestock cluster, on average, exhibited the potential for resistance to at least six antibiotic classes. The aminoglycoside, beta-lactam, fluoroquinolone, and sulfonamide antibiotic groups consistently displayed resistance across all pig farming clusters. Among the livestock clusters, cluster 9 had the highest potential for resistance to *E. coli*, encompassing eight antibiotic classes (**Fig. 1**).

Diversity and distribution of ARGs

The genome sequence analysis of *E. coli* derived from fecal samples in pig farming clusters in Banten Province identified 51 distinct ARGs. Each livestock cluster harbors a minimum of 10 ARGs, with clusters 3 and 6 exhibiting the minimum and maximum, respectively, with 23 ARGs (**Fig. 2**). The distribution of ARGs across antibiotic classes included 15, 10, nine, eight, three, two, and one from the aminoglycoside group, sulfonamide group, tetracycline group, beta-lactam group, chloramphenicol group, macrolide and fluoroquinolone groups, and the lincosamide and phosphonic groups, respectively. In particular, the *ant(3'')-Ia* and *qnrS1* genes are prevalent, being present in all pig farming clusters in Banten Province (**Table 2**).

Plasmid profile

The prevalence of ARGs in *E. coli* from fecal samples within pig farming clusters in Banten Province is predominantly associated with plasmids rather than chromosomes. On average, 89.4% of the identified ARGs in the *E. coli* pig farming cluster were plasmid-borne, with values ranging from 43% to 97%. Clusters 4 and 7 exhibited the highest percentage of ARGs originating from plasmids, recording 97%. In contrast, ARGs sourced from chromosomes in *E. coli* isolates from pig farming clusters averaged only 10.6%, ranging from 3% to 57%. The highest percentage of chromosome-associated ARGs was observed in cluster 13 farms (**Table 3**).

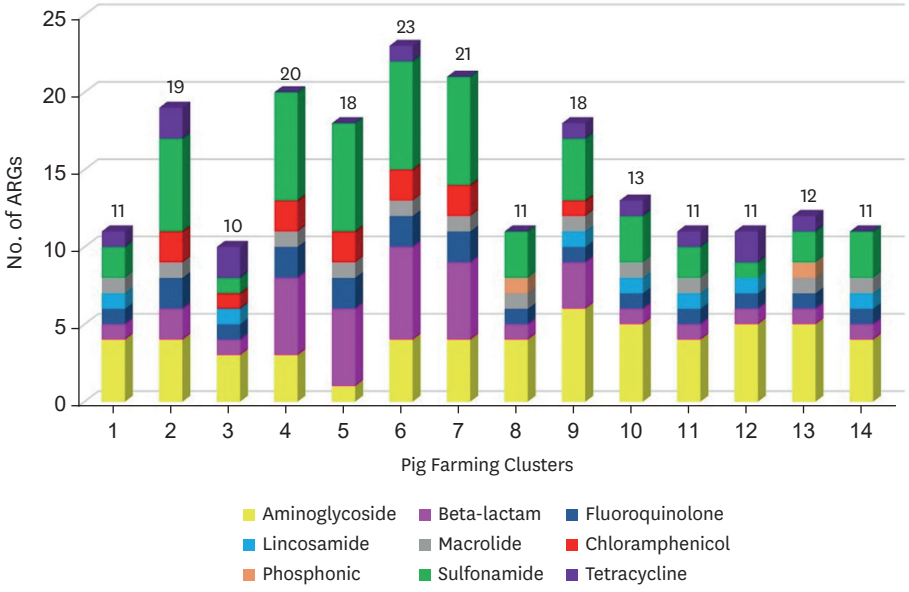

Fig. 2. Distribution of ARGs in *E. coli* across each cluster of pig farms in Banten Province.
ARG, antibiotic resistance gene.

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Table 2. ARGs profile of *E. coli* genomes in pig farm clusters in Banten Province

Antibiotic class	ARG	Pig farming cluster														●	Percentage (%)
		1	2	3	4	5	6	7	8	9	10	11	12	13	14		
Aminoglycoside	*aph(6)-Id*	●	x	x	x	x	x	x	x	●	●	●	●	x	●	6	42.9
	*aph(6)-Id**	x	x	x	x	x	x	x	●	x	x	x	x	●	x	2	14.3
	aph(3")-Ib	●	x	x	x	x	x	x	x	●	●	●	●	x	●	6	42.9
	*aph(3")-Ib**	x	x	x	x	x	x	x	●	x	x	x	x	●	x	2	14.3
	*aph(3')-Ia**	x	x	x	x	x	x	x	●	x	x	x	x	●	x	2	14.3
	aac(3)-Iid	●	x	x	x	x	x	x	x	●	●	●	●	x	●	6	42.9
	*ant(3")-Ia**	x	x	x	x	x	x	x	x	x	x	x	x	●	x	1	7.1
	ant(3")-Ia	●	●	●	●	●	●	●	●	●	●	●	●	●	●	14	100.0
	ant(3")-Ii	x	●	x	●	x	●	●	x	x	x	x	x	x	x	4	28.6
	aac(6')-Iid	x	●	x	●	x	●	●	x	x	x	x	x	x	x	4	28.6
	aadA2	x	●	●	x	x	●	●	x	x	x	x	x	x	x	4	28.6
	aac(3)-Ib	x	x	●	x	x	x	x	x	x	x	x	x	x	x	1	7.1
	aadA17	x	x	x	x	x	x	x	x	x	●	x	●	x	x	2	14.3
	aadA12	x	x	x	x	x	x	x	x	●	x	x	x	x	x	1	7.1
	aadA5	x	x	x	x	x	x	x	x	●	x	x	x	x	x	1	7.1
Beta-lactam	*blaCTX-M-55*	●	x	x	x	x	x	x	●	●	x	●	●	●	●	7	50.0
	blaOXA-10	x	●	x	●	●	●	●	x	x	x	x	x	x	x	5	35.7
	blaTEM-1B	x	●	●	●	●	●	●	x	●	x	x	x	x	x	7	50.0
	blaTEM-105	x	x	x	●	●	●	●	x	x	x	x	x	x	x	4	28.6
	blaTEM-141	x	x	x	●	●	●	●	x	x	x	x	x	x	x	4	28.6
	blaCTX-M-15	x	x	x	●	●	●	●	x	x	●	x	x	x	x	5	35.7
	blaTEM-122	x	x	x	x	x	●	x	x	x	x	x	x	x	x	1	7.1
	blaTEM1C	x	x	x	x	x	x	x	x	●	x	x	x	x	x	1	7.1
Fluoroquinolone	*qnrS1*	●	●	●	●	●	●	●	●	●	●	●	●	●	●	14	100.0
	qnrVC4	x	●	x	●	●	●	●	x	x	x	x	x	x	x	5	35.7
Lincosamide	*lnu (F)*	●	x	●	x	x	x	x	x	●	●	●	●	x	●	7	50.0
Macrolide	*mef(B)*	x	●	x	●	●	●	●	x	x	x	x	x	x	x	5	35.7
	mph (A)	●	x	x	x	x	x	x	●	●	●	●	x	●	●	7	50.0
Chloramphenicol	*catA1*	x	x	x	x	x	x	x	x	●	x	x	x	x	x	1	7.1
	catB2	x	●	x	●	●	●	●	x	x	x	x	x	x	x	5	35.7
	cmlA1	x	●	●	●	●	●	●	x	x	x	x	x	x	x	6	42.9
Phosphonic	*fosA4*	x	x	x	x	x	x	x	●	x	x	x	x	●	x	2	14.3
Sulfonamide	*sul1*	x	●	x	●	●	●	●	x	●	x	x	x	x	x	6	42.9
	sul2	●	●	x	●	●	●	●	●	●	●	●	●	x	●	12	85.7
	*sul2**	x	x	x	x	x	x	x	●	x	x	x	x	●	●	3	21.4
	sul3	x	●	●	●	●	●	●	x	x	x	x	x	x	x	6	42.9
	dfrA1	x	x	x	●	●	●	●	x	x	x	x	x	x	x	4	28.6
	*dfrA1**	x	x	x	x	x	x	x	x	x	●	x	x	x	x	1	7.1
	dfrA12	x	●	x	●	●	●	●	x	x	x	x	x	x	x	5	35.7
	dfrA14	●	●	x	●	●	●	●	●	●	●	●	x	●	●	12	85.7
	dfrA16	x	●	x	●	●	●	●	x	x	x	x	x	x	x	5	35.7
	dfrA17	x	x	x	x	x	x	x	x	●	x	x	x	x	x	1	7.1
Tetracycline	*tet(A)*	x	x	x	x	x	●	x	x	x	x	x	x	x	x	1	7.1
	*tetA**	x	x	x	x	x	x	x	x	x	x	x	●	x	x	1	7.1
	tet(B)	x	x	x	x	x	x	x	x	●	x	x	x	x	x	1	7.1
	*tet(B)**	x	x	●	x	x	x	x	x	x	x	●	x	●	x	3	21.4
	tet (M)	x	x	●	x	x	x	x	x	x	x	x	x	x	x	1	7.1
	tetO	x	x	x	x	x	x	x	x	x	●	x	x	x	x	1	7.1
	tetO(34)	●	x	x	x	x	x	x	x	x	x	x	x	x	x	1	7.1
	tetX	x	●	x	x	x	x	x	x	x	x	x	●	x	x	2	14.3
	*tetX**	x	●	x	x	x	x	x	x	x	x	x	x	x	x	1	7.1

ARG, antibiotic resistance gene.
●, ARG detected; x, ARG undetected.

DISCUSSION

This research conducted a WGS analysis of *E. coli* isolates obtained from fecal samples in pig farming clusters in Banten Province, building upon prior investigations [12]. In particular,

Table 3. Percentage of plasmids carrying ARGs in *E. coli* from fecal samples of pig farm clusters in Banten Province

Farm cluster	Plasmid	Chromosome
1	11 (91.7)	1 (8.3)
2	25 (92.6)	2 (7.4)
3	11 (84.6)	2 (15.4)
4	33 (97.0)	1 (3.0)
5	31 (94.0)	2 (6.0)
6	42 (95.5)	2 (4.5)
7	31 (97.0)	1 (3.0)
8	7 (54.0)	6 (46.0)
9	28 (96.5)	1 (3.5)
10	13 (86.7)	2 (13.3)
11	11 (77.0)	2 (33.0)
12	10 (91.0)	1 (9.0)
13	6 (43.0)	8 (57.0)
14	11 (92.0)	1 (8.0)
Average	89.4	10.6

Values are presented as number (%).

WGS research on *E. coli* derived from pig farm samples in Banten Province marked the pioneering effort in Indonesia, providing crucial data for developing preventive and control measures against antibiotic resistance. WGS applications offered comprehensive insights into the initial emergence and spread of antibiotic resistance, facilitating predictions regarding likely antibiotic resistance profiles and serving as a foundation for developing effective policies to control antibiotic resistance [8].

Phylogenetic analysis of *E. coli* in pig farming clusters in Banten Province revealed three distinct phylogenetic groups: Group 1 (cluster 1), Group 2 (cluster 4), and Group 3. Within phylogenetic Group 3, pig farming clusters were further categorized into two lineages (clades): the first clade (cluster 6) and the second clade (clusters 2, 5, 11, 7, 14, 3, 10, 8, 13, 12, and 9). The second clade was subsequently divided into two sub-clades: the first sub-clade (clusters 2 and 5) and the second sub-clade (clusters 11, 7, 14, 3, 10, 8, 13, 12, and 9). Close relationships were observed between clusters 9 and 12, clusters 8 and 13, and clusters 3 and 14. Interestingly, the study findings indicated that *E. coli*, which are closely related phylogenetically, do not always have similar distribution and resistance patterns (**Fig. 1**).

E. coli isolated from fecal samples in a pig farming cluster in Banten Province exhibited resistance to nine classes of antibiotics, including those crucial for human medicine. Dominant antibiotic resistance was observed in the aminoglycosides, beta-lactams, fluoroquinolones, sulfonamides, and macrolides groups in the pig farming cluster. Consistent with Peng et al. [23], *E. coli* isolated from pig farms in China displayed resistance to multiple antibiotic classes, including sulfonamides, tetracyclines, fluoroquinolones, macrolides, and beta-lactams. Similarly, Carhuaricra et al. [24] reported prevalent resistance in *E. coli* from fecal samples in pig farms in Lima, Peru, particularly to tetracyclines, sulfonamides, aminoglycosides, chloramphenicol, beta-lactams, and fluoroquinolones.

Even more concerning, all *E. coli* isolated from the fecal samples in the pig farm cluster showed multidrug resistance. Each livestock cluster exhibited resistance to at least six classes of antibiotics, with the highest achieving resistance to eight classes of antibiotics, as observed in cluster 9. The elevated prevalence of multidrug resistance in *E. coli* from pig farm samples has been reported consistently [23, 25]. Zhang et al. [25] reported a rise in MDR *E. coli* in pig farms that they attributed to the excessive and uncontrolled use of antibiotics. This

poses a serious risk because high levels of MDR *E. coli* in pig farms can disseminate into the environment, escalating the risk of transmission to humans and posing a significant threat to public health [26]. The heightened level of multidrug resistance raises serious concerns regarding the limited availability of effective antibiotics for treating bacterial diseases, diminishing the ability to combat infectious diseases in humans and animals [27].

Tetracycline antibiotics (oxytetracycline and tetracycline), beta-lactams (amoxicillin and penicillin), and sulfonamides (sulfadimethypyrimidine sodium, sulfadiazine sodium, sulfadimidine sodium, and sulfamerazine sodium) are commonly used in pig farming in Indonesia [28]. The majority of antibiotics in pig farms are used for treatment (55.21%), disease prevention (42.71%), and production enhancement (2.08%) [28]. Previous research also reported the use of sulfonamide antibiotics (sulfamonomethoxine and sulfamethoxazole), tetracyclines (chlortetracycline, doxycycline, and oxycycline), fluoroquinolones (enrofloxacin), macrolides (kitasamycin tartrate), and beta-lactams (cefotaxime, penicillin, and amoxicillin) in pig farming for growth promotion or disease treatment [23]. A previous study reported the highest percentage of feed containing antibiotics in pigs (55.4%) compared to chickens (42.2%), quail (18.9%), and ducks (9.2%). The most commonly added antibiotics in swine feed formulations were Bacitracin (24.8%), chlortetracycline (23.9%), and florfenicol (17.4%) [4].

Phylogenetic analysis of *E. coli* in the pig farming clusters in Banten Province revealed significant variations in antibiotic resistance levels and carried ARGs despite being the same breed and closely related. For example, livestock clusters 9 and 12, clusters 8 and 13, and clusters 3 and 14, although closely related, displayed substantial differences in antibiotic resistance. In particular, livestock cluster 9 exhibited resistance to macrolide and chloramphenicol antibiotics, while cluster 12 did not. Furthermore, the ARG diversity of cluster 9 included 18 ARGs. In contrast, cluster 12 carried 11 ARGs (**Fig. 1**). This variability may be influenced by the role of plasmids and other MGEs, which predominantly participate in carrying MDR genes and horizontally transferring genes between bacterial species and across unrelated species [7]. Consequently, the proliferation of bacteria carrying MDR genes extends beyond livestock to impact the environment and human populations.

The genome sequence analysis of *E. coli* in this study revealed a high diversity of ARGs across all pig farming clusters in Banten Province. Fifty-one ARGs from nine classes of antibiotics were identified in *E. coli* isolates, with each cluster carrying a minimum of 10 ARGs (cluster 3) and the highest count being 23 ARGs (cluster 6). Aminoglycoside class ARGs were the most frequently detected (15 ARGs), followed by sulfonamide class (10 ARGs), tetracycline class (9 ARGs), and beta-lactam class (8 ARGs) (**Fig. 2**).

Genes from the aminoglycoside class, such as *ant(3'')-Ia* (100%), *aph(3'')-Ib* (42.9%), *aph(6)-Id* (42.9%), and *aac(3)-Iid* (42.9%), were dominantly present in *E. coli* clusters from pig farms (**Table 2**). The same thing was also reported elsewhere [24,29]. In particular, the *ant(3'')-Ia* gene was found in all pig farming clusters, frequently associated with the cassettes gene in class 1 integrons, and is the most commonly found ARG in Gram-negative bacteria globally [7,30]. The *aph(6)-Id* and *aph(3'')-Ib* genes, which are associated with streptomycin resistance, are widespread in *E. coli*, and are often linked to unique mobile elements and globally distributed in pigs [31]. The *ant(3'')-Ia*, *aph(3'')-Ib*, *aph(6)-Id*, and *aac(3)-Iid* genes play a role in the enzymatic inactivation resistance mechanism, which can inactivate aminoglycosides by modifying the molecules so that they cannot reach or binding to the target site. The *ant(3'')-Ia* gene

produces the nucleotidyltransferase enzyme. The *aph(3'')-Ib* and *aph(6)-Id* genes produce the phosphotransferase enzyme, while the *aac(3)-Iid* produces the acetyltransferase enzyme [7].

This study revealed ESBL-producing *E. coli*, which is a global health concern [24,29]. The predominant beta-lactam ARGs included *blaCTX-M-55* (50% cluster) and *blaTEM1B* (50% cluster), followed by *blaCTX-M-15* (35.7%) and *blaOXA-10* (35.7%) (**Table 2**). Similar findings were reported in other studies, with *blaCTX-M-55* dominating in pigs in Vietnam [32] and *blaTEM1B* being prevalent in pig farms in Peru [24]. The *blaCTX-M* gene in *E. coli* is distributed widely in livestock (pigs and poultry), meat, vegetables, and humans. The *blaCTX-M* gene has several variants including *blaCTX-M-1*, *blaCTX-M-15*, *blaCTX-M-55*, *blaCTX-M-9*, *blaCTX-M-14*, *blaCTX-M-27*, and *blaCTX-M-65* [32,33].

From the fluoroquinolone class, two ARGs, *qnrS1* (100%) and *qnrVC4* (35.7%), were detected (**Table 2**). The *qnrS1* gene, detected in all pig farming clusters, is the most commonly found gene in *E. coli* from pig populations [7,34]. The gene is associated with plasmid-mediated quinolone resistance (PMQR), protecting DNA from quinolone binding. These genes are often found on IncX1 and IncN plasmids, facilitating the spread of ARGs to various animal species and environments [35]. PMQR-mediated genes have been reported in the last two decades, resulting in an even more complex genetic backbone of fluoroquinolone resistance [7,34]. The high prevalence of *qnrS1* poses challenges in controlling fluoroquinolone resistance, particularly considering the classification of fluoroquinolones as critically important antibiotics for human medicine [27]. Their use in livestock raises concerns about the cross-selection of genetic determinants of resistance to antimicrobials used in human medicine, posing a severe threat to public health [36].

The *sul2* (85.7%) and *dfrA14* (85.7%) genes were the most dominant genes detected from the sulfonamide class in this study (**Table 2**). The *sul1*, *sul2*, and *sul3* genes are distributed widely in *E. coli* from various animal species worldwide, and *sul2* is the most dominant ARG in pig farms. Several studies have reported that the *sul1*, *sul2*, and *sul3* genes are often located on plasmids, including plasmids that also contain other ARGs (MDR) [31,37]. The *sul2* gene is often associated with the streptomycin resistance gene *strA-strB* [7]. Similarly, *sul1* is often found with other ARGs in gene cassettes in variable regions of class 1 integrons and MDR plasmids carrying ESBL genes [37]. The *sul3* gene is also associated with the macrolide resistance gene *mef(B)* and class 1 integrons [7]. IncFII is the dominant type in *sul2*-carrying plasmids, while IncI1 is the most common type in *sul1*- and *sul3*-carrying plasmids [37]. The *dfrA14* gene has been identified in *E. coli* from pigs in various countries worldwide [30]. Previous studies showed that the *dfrA14* gene in *E. coli* originating from animals and food products from an animal origin is related to integrons. The functionally active *dfrA14* gene from *E. coli* in food products of animal origin was found outside the integron but was inserted into the plasmid-borne *strA* gene [38].

Tetracycline, an antibiotic used extensively in animals, constitutes 37% of the total antimicrobial agent sales for animals in 25 European Union and European Economic Area countries [39]. The indiscriminate use of this antibiotic exerts selective pressure on bacteria, particularly *E. coli*, leading to the development of resistance to tetracycline antibiotics through various tetracycline resistance genes (*tet* genes). *tetB** (21.4%) and *tetX* (14.3%) were predominant among the *tet* genes identified in this study. The *tetA* and *tetB* genes, prevalent in *E. coli* from animal samples, particularly pigs, are part of conjugative and nonconjugative transposons, such as Tn1721 (*tetA*) and Tn10 (*tetB*), integrated into plasmids. Single *E. coli* isolates often exhibit multiple *tet* genes resulting from various *tet* genes on plasmids or other

MGEs acquired at different times and under different conditions. In addition, *E. coli* plasmid-borne *tet* genes can combine with other ARGs from diverse antibiotic classes, resulting in MDR plasmids [7]. MDR plasmids were reported in *E. coli* from various animal species in various countries, such as the T078 plasmid carrying the *qnrS1*, *blaCTX-M-14*, *blaTEM-1*, *floR*, and *tetA* genes in *E. coli* isolated from pigs in China [40].

The *tetX* gene, previously unreported in Indonesian pigs, was first identified in this study. The *tetX* gene, commonly with the *tetX4* variant, is frequently associated with plasmids, facilitating the exchange of genetic information among bacteria. The plasmid-mediated *tetX* gene (*tetX4* variant) has been found in animal-origin samples globally (pigs, ducks, geese, broilers, cows, freshwater fish, shrimp, and migratory birds), with pigs being the predominant source [41]. Studies from China revealed the emergence of tigecycline resistance mediated by the *tetX4* plasmid in *E. coli* isolates. The highly transmissible IncQ1 plasmid carrying *tetX4* can mobilize and stabilize in clinical and laboratory strains of Enterobacteriaceae bacteria. *TetX4*-positive *E. coli* strains, also containing *mcr-1*, are widespread in pigs, poultry, soil, and dust samples in China [42]. Plasmid-mediated *tetX4* compromises the efficacy of all tetracycline group antibiotics, including tigecycline, which is a last-resort antibiotic for complicated bacterial infections caused by MDR Gram-negative and Gram-positive bacteria [43].

Chloramphenicol, an antibiotic prohibited in the Indonesian livestock industry according to Minister of Agriculture Regulation No. 14/PERMENTAN/PK.350/5/2017 [44] and Minister of Agriculture Decree No. 9736/PI.500/F/09/2020 [45], displayed resistance in seven pig farming clusters in this study. *E. coli* in these clusters carried three ARGs: *cmlA1*, *catB2*, and *catA1*. *cmlA1* was the most prevalent (42.9%), followed by *catB2* (35.7%) and *catA1* (7.1%). Peng et al. [23] reported similar findings in China, where chloramphenicol-resistant *E. coli* (*cmlA*, *floR*) persisted in pig farms despite the antibiotic ban since 2002 [46], possibly because of the use of florfenicol, a related antibiotic, in pig farming [4,47]. AbuOun et al. [29] also reported the presence of *catA1*, *catA6*, *cml*, and *floR* genes in pig farms in the United Kingdom.

In pig farming clusters of Banten Province, the majority of ARGs in *E. coli* were plasmid-borne (89.4%) rather than chromosomal (10.6%). Clusters 4 and 7 exhibited the highest percentage of ARGs originating from plasmids (97%) (**Table 3**). Plasmids play a crucial role in the horizontal transfer of ARGs with conjugation between bacterial species, facilitating the spread of multidrug resistance genes [48]. The IncF plasmid type, prevalent in humans and animals, particularly in *E. coli*, is a major contributor to this dissemination, carrying resistance and virulence determinants [49]. This plasmid was identified most widely during the pandemic, carrying MDR genes, virulence of the *E. coli* O25:H4-ST131 clone, and *blaCTX-M-15* gene [35]. IncHI1 and IncHI2 plasmids are also frequently associated with resistance to antibiotics, such as sulfonamides, aminoglycosides, tetracyclines, streptomycin, and cephalosporins [50]. These plasmids aid in the spread of specific ARGs and contribute to the selection and persistence of other ARGs [7].

The challenge in controlling antibiotic resistance lies in plasmid-borne ARGs, which contribute significantly to the spread of resistance determinants and undistinguishable plasmids identified in bacterial strains from very distant geographical areas, usually without any clear epidemiological link. Plasmids, common in natural bacteria, often carry multiple linked genetic determinants, simultaneously conferring resistance to several antibiotic classes. Furthermore, plasmids harbor virulence factors and addiction systems, enhancing their stability and persistence in bacterial hosts across diverse environmental conditions [35,48].

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