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Determining the prevalence and genetic diversity of plasmid-mediated sulfonamide resistance in *Escherichia coli* from commercial broiler samples

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ABSTRACT Sulfonamides are commonly used antibacterials in commercial poultry, contributing toward the development of multidrug-resistant (**MDR**) phenotypes among *Escherichia coli* and that has emerged as global concern. The current study aimed to assess the sulfonamide resistance among isolated *E. coli* strains among commercial broilers. The bacterial strains were identified from fecal samples ($n = 100$) using selective media, followed by initial identification based on biochemical profiles. The susceptibility was determined by measuring the minimum inhibitory concentration (**MIC**) against sulfamethoxazole. The study also evaluated mobile genetic elements (**MGEs**), the mediators of antibiotic resistance, by amplification of plasmid DNA using specific primer PCR. Additionally, the isolates were subjected to multilocus sequence typing (**MLST**) analysis to investigate the genetic diversity among *E. coli* carrying sulfonamide resistance genes. The results revealed that 58% (58/100) *E. coli* strains were resistant to sulfonamides, with 36.20% (21/58) of the strains exhibiting an MIC breakpoint ≥512 μg/mL. PCR analysis showed that 42.85% (9/21) of the strains harbored the *sul-1* gene, while 38.09% (8/21) carried the *sul-2* gene, and 19.04% (4/21) had both genes. No isolate showed the presence of the *sul-3* gene. Furthermore, class 1 and class 2 integrons were identified among 80.95% (17/21) and 19.04% (4/21) of the strains, respectively. MLST analysis confirmed that the strains belonged to sequence types (**STs**) including ST1638, ST155, ST48, ST350, ST23, ST156, and ST746. These findings underscore the diversity among *E. coli* strains in commercial poultry, which poses a significant risk.

Key words: *Escherichia coli*, sulfonamide, mobile genetic element (MGE), resistance, broiler

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INTRODUCTION

Escherichia coli is a natural inhabitant and/ or opportunistic pathogen that resides in the gastro-intestinal tract of humans and animals (Nawaz et al., 2019; Alagawany et al., 2020; Rafique et al., 2020; Alagawany et al., 2022). Among commercial poultry, the avian pathogenic *E. coli* (**APEC**) strains are responsible for numerous infections, including colibacillosis, peritonitis, pericarditis, coligranuloma, synovitis, osteomyelitis, salpingitis, and cellulitis (De Carli et al., 2015). Colibacillosis leads to the severe illness and significant economic losses among commercial poultry (Karczmarczyk et al., 2011).

For the treatment of *E. coli* infections in food animals, sulfonamides, streptomycin, cephalosporins, fluproquazone, tetracycline, ampicillin, and colistin are frequently used (Suojala et al., 2013; Ibrahim et al., 2019). The utilization of several antimicrobials for growth stimulation has been restricted in developed countries, whereas, it remains common in other countries for growth promotion or for therapeutic purposes (Azam et al., 2020; Van et al., 2020). In Pakistan, approximately 568 tons of antimicrobials are estimated to be used annually in poultry (Mohsin et al., 2019). It is common practice to use antibiotics excessively and prophylactically on food animals in an effort to boost production and maintain animal health (Hao et al., 2014; Alhaji and Isola, 2018;

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Umair et al., 2022). However, antimicrobial resistance (**AMR**) is responsible for the significant risk to food safety and has the potential to disseminate resistance among humans (Manyi-Loh et al., 2018; Khan et al., 2021; Zulqarnain et al., 2021). Transmission of AMR *E. coli* strains have been described among humans-poultry-environment (Aworh et al., 2019). Improper use of antibiotics is linked to the development and distribution of AMR, as highlighted in several reports (Marshall and Levy, 2011; Zhuge et al., 2019). The presence of *E. coli*, in various hosts, that is, cattle, humans and poultry, has contributed to the transmission of resistance genes between humans and animals (O'Brien, 2002; Nawaz et al., 2021). MGEs including integrons and plasmids are the elements associated with dissemination of resistance genes among bacterial communities (Livermore, 2003; Mahmood et al., 2022).

Sulfonamides are synthetic antimicrobials used as bacteriostatic drugs against bacterial infections (van Duijkeren et al., 2018). Sulfonamide resistance is developed due to dihydropteroate synthase (**DHPS**), the *sul* gene (Wang et al., 2014). The dissemination of antibiotic resistance is either vertical or horizontal (Ben et al., 2017) as the *sul*-genes are located on chromosomes as well as on plasmids. MGEs facilitate the translocation of sulfonamide-resistant genes between plasmids and chromosomal DNA (Rehman et al., 2017). Moreover, plasmids carrying sulfonamide-resistant genes can disseminate among similar or different bacterial pathogens by transformation or conjugation (Wu et al., 2010). Previously, different sulfonamide-resistant genes (*sul-1, sul-2*, and *sul-3*) have been reported (Sköld, 2001; Perreten and Boerlin, 2003) which are located on conjugative plasmids and class 1 integrons (Radstrom et al., 1991; Hammerum et al., 2006; Sagor et al., 2022). Altogether, the primary objective of the current study was to determine the genetic diversity and prevalence of *E. coli*, harboring plasmid-mediated sulfonamide resistance genes, isolated from commercial broilers.

MATERIALS AND METHODS

Bacterial Strains

Fecal samples ($n = 100$) were collected from 5 commercial broiler farms in Faisalabad, Pakistan. The collection process ensured aseptic conditions, and the samples were promptly transported to the laboratory while maintaining controlled temperature conditions (Idrees et al., 2011). The samples were inoculated on bacterial culture media and were subjected to confirmation using commercially available API 20 kit (BioMerieux, France) as described by Jamil et al. (2022).

Minimum Inhibitory Concentration

The antibiotic susceptibility was conducted using broth microdilution according to the guidelines of Clinical and Laboratory Standards Institute (CLSI-2022) against sulfamethoxazole, as recently described by Jamil et al. (2022).

Detection of Sulfonamide Resistance Genes and Integrons

The extracted plasmid DNA was subjected to PCR analysis to identify genes (*sul-1, sul-2*, and *sul-3*). The used primers for amplifications of resistance genes determinate were listed in Table 1. The PCR amplification conditions were followed as recently described by Jamil et al. (2022) using T100 Thermal Cycler from Bio-Rad. The presence of class 1, class 2, and class 3 integrons were also detected. To ensure the quality of the PCR analysis, positive and negative controls were included. About 1.5% (w/v) agarose gel was used for electrophoresis of amplicons using QA-Agarose from MP Biomedical (MP Biomedical, Birmingham, United Kingdom) along with 100 bp DNA ladder (Invitrogen, Birmingham, United Kingdom). The gel was visualized by Uvitech, UK (Zhu et al., 2013).

Multilocus Sequence Typing

For the sulfonamide-resistant strains carrying *sul-1* and *sul-2* genes, multilocus sequence typing (**MLST**) was conducted as recently described by Jamil et al. (2022). The obtained sequences were aligned using the ClustalW Algorithm in MEGA software, and allelic numbers were assigned accordingly. To determine the allelic profiles and sequencing types (**STs**) of each

Table 1. Primers used for amplification of sulfonamides resistance genes and integrons determinants.

Target gene	Primers	Sequences	Annealing temperature	Product size	References
Sul-1	Sul-1-(F)	CGG CGT GGG CTA CCT GAA CG	55	433	Kozak et al. (2009a)
	Sul-1-(R)	GCC GAT CGC GTG AAG TTC CG			
Sul-2	Sul-2-(F)	CGG CAT CGT CAA CAT AAC CT	56	721	Kozak et al. (2009a)
	Sul-2-(R)	TGT GCG GAT GAA GTC AGC TC			
Sul-3	Sul-3-(F)	CAA CGG AAG TGG GCG TTGTGGA	55	244	Kozak et al. (2009a)
	Sul-3-(R)	GCT GCA CCA ATT CGC TGA ACG			
Int-1	Int-1-(F)	GCC TTG CTG TTC TTC TAC GG	55	565	Levesque et al. (1995)
	Int-1-(R)	GAT GCC TGC TTG TTC TAC GG			
Int-2	Int-2-(F)	AAG CAG ACT TGA CCTGA	55	565	White et al. (2001)
	Int-2-(R)	CAC GGA TAT GCG ACA AAA AGGT			
Int-3	Int-3-(F)	AGTGGGTGGCGAATGAGTG	55	484	Goldstein et al. (2001)
	Int-3-(R)	TGTTCTTGTATCGGCAGGTG			

Table 2. Determination of minimum inhibitory concentration (MIC) of *Escherichia coli* isolates.

		No. of isolates at MIC (μg/mL) of											
Antimicrobial agents	Breakpoints	≤0.25	0.5	1	2	4	8	16	32	64	128	≥256	≥512
Sulfamethoxazole	≥512	-	-	-	-	3	2	3	7	5	6	11	21

isolate, the Entero-base Database was consulted as recently described by Jamil et al. (2022).

RESULTS

Bacterial Strains

Out of the total 100 fecal samples analyzed, 58% (58/100) tested positive for the presence of *E. coli* based on cultural and morphological characteristics. This determination was further supported by biochemical profiling methods.

Minimum Inhibitory Concentration

Out of total 58 *E. coli* strains, 36.20% (21/58) showed resistance to sulfamethoxazole. On the other hand, 63.79% (37/58) of the strains were negative for sulfonamide resistance. The minimum inhibitory concentration (**MIC**) breakpoint for sulfonamide was set at ≥512 μg/mL. Detailed results are described in Table 2.

Detection of Sulfonamide Resistance Genes and Integron

The PCR results revealed that out of the 21 tested strains, 42.85% (9/21) were found to harbor the sul-1 gene, while 38.09% (8/21) were identified as positive for the sul-2 gene. Additionally, 19.04% (4/21) of the strains coharbored both the *sul-1* and *sul-2* genes. No isolates showed the presence of the *sul-3* gene.

In terms of integrons, class 1 integrons were detected in 80.95% (17/21) of the strains, while class 2 integrons were found in 19.04% (4/21) of the strains, whereas *sul*-1 (9/21) 42.85%, *sul*-2 (8/21) 38.09% and coharbored *sul*-1 and *sul*-2 genes (4/21) 19.04% were identified, as described in Table 3. The distribution of sulfonamide resistance gene and integrons is described in Table 4.

Multilocus Sequence Typing

The MLST analysis revealed that the *sul-1* and *sul-2* positive isolates belonged to various sequence types (**STs**) with different clonal complex (**CC**) associations. Specifically, ST1638 was identified in 5 strains without any clonal complex association, and these strains carried both *sul-1* and *sul-2* genes. ST155 was associated with CC155 and found in 4 strains carrying the *sul-1* gene. ST48 was reported in 3 strains and carried the *sul-2* gene. ST350 was identified in 3 strains and carried the *sul-2* gene, while also coharboring the *sul-1* and *sul-2* genes. ST23, ST156, and ST746 were each reported in 6 strains, but the specific genes they carried were not specified. These findings demonstrate the diversity of sequence types and gene combinations observed among the *sul-1* and *sul-2* positive isolates.

DISCUSSION

Antimicrobial resistance (**AMR**) is an emerging global health concern due to irrational and extensive use of antibiotics among food producing animals particularly as growth promoters or therapeutic purposes that has contributed to the development of resistant strains (Jamil et al., 2022). The sulfonamides are not commonly prescribed in human medicine in many countries; however, these are widely used in veterinary settings due to the lower cost (Jiang et al., 2019). Previously published data showed that approximately 50 to 80% of the antibiotics are used in livestock (Cully, 2014).

In veterinary settings, commonly used antibiotic groups include sulfonamides (12%), tetracyclines (32%), penicillin (26%), polymyxins (5%), macrolides (7%), and aminoglycosides (5%) (EMA, 2018; Majewski et al., 2020). In Pakistan, *E. coli* strains are frequently involved in contaminating poultry products, and they often exhibit resistance due to the regular use of antibiotics, particularly sulfonamides. Poultry birds play significant role in spread of diverse bacterial strains that could disseminate resistance against amikacin, amoxicillin, clindamycin, ciprofloxacin, chloramphenicol, and

Table 3. Sequence type distribution and clonal complex details among sulfonamide resistant *Escherichia coli* strains isolated from commercial broilers.

Clonal complex (CC)	Sequence types (STs)	Integrase class 1 and Integrase class 2
−−−	1638 (5/21), 23.80%	*sul*-1 (9/21) 42.85% *sul*-2 (8/21) 38.09% Coharbored *sul*-1 and *sul*-2 genes (4/21) 19.04%
155	155 (4/21), 19.04%	Integrase Class 1 (17/21) 80.95% Integrase Class 2 (4/21) 19.04%
10 350	48 (3/21), 14.28% 350 (3/21), 14.28%	Association of Integrase Class 1 & Class 2 with *sul*-1 and *sul*-2 Genes
156 23 −−−	156 (2/25), 9.52% 23 (2/25), 9.52% 746 (2/25), 9.52%	Integrase Class 1+ *sul*-1 gene (8/21) 38.09% Integrase Class 1+ *sul*-2 gene (5/21) 23.80% Integrase Class 2 + *sul*-1 gene (1/21) 4.76% Integrase Class 2 + *sul*-2 gene (3/21) 14.28% Integrase Class 1+ *sul*-1 +*sul*-2 genes (4/21) 19.04%

Table 4. MLST of *Escherichia coli* isolates harboring *sul*-1 gene and *sul*-2 genes.

Isolate ID	Specimen	Sequence type (ST)	Clonal complex (CC)	Resistant genes	Integrase
Sr-Ec-1	Fecal	ST-155	CC-155	*sul*-1	*Int*1
Sr-Ec-2	Fecal	ST-1638	___	*sul*-1	*Int*1
Sr-Ec-3	Fecal	ST-23	CC-23	*sul*-1	*Int*1
Sr-Ec-4	Fecal	ST-23	CC-23	*sul*-1	*Int*1
Sr-Ec-5	Fecal	ST-155	CC-155	*sul*-1	*Int*2
Sr-Ec-6	Fecal	ST-155	CC-155	*sul*-1	*Int*1
Sr-Ec-7	Fecal	ST-155	CC-155	*sul*-1	*Int*1
Sr-Ec-8	Fecal	ST-156	CC-156	*sul*-1, *sul*-2	*Int*1
Sr-Ec-9	Fecal	ST-156	CC-156	*sul*-1, *sul*-2	*Int*1
Sr-Ec-10	Fecal	ST-1638	___	*sul*-1	*Int*1
Sr-Ec-11	Fecal	ST-1638	___	*sul*-1	*Int*1
Sr-Ec-12	Fecal	ST-48	CC-10	*sul*-2	*Int*1
Sr-Ec-13	Fecal	ST-1638	___	*sul*-2	*Int*2
Sr-Ec-14	Fecal	ST-1638	___	*sul*-2	*Int*2
Sr-Ec-15	Fecal	ST-48	CC-10	*sul*-2	*Int*1
Sr-Ec-16	Fecal	ST-48	CC-10	*sul*-2	*Int*1
Sr-Ec-17	Fecal	ST-350	CC-350	*sul*-2	*Int*2
Sr-Ec-18	Fecal	ST-350	CC-350	*sul*-1, *sul*-2	*Int*1
Sr-Ec-19	Fecal	ST-350	CC-350	*sul*-1, *sul*-2	*Int*1
Sr-Ec-20	Fecal	ST-746	___	*sul*-2	*Int*1
Sr-Ec-21	Fecal	ST-746	___	*sul*-2	*Int*1

Sr-Ec = sulfonamide resistant *E. coli; Int* = integrons class.

colistin, etc. (Box et al., 2005; Khan et al., 2021; Zulqarnain et al., 2021).

In this study, a total of 58% (58/100) fecal specimens were found positive for *E. coli*. This prevalence rate is significantly high as compared recently published data 16.8%) in Nigeria (Aworh et al., 2021). A study from Korea reported significantly lower prevalence rate of 4.9% (Lee et al., 2009). Whereas one of the studies conducted in India reported prevalence of *E. coli* as 66.66% (Pandey et al., 2016). About 66.66% prevalence of *E. coli* was also reported in Pakistan (Zulqarnain et al., 2021). The variations in these studies can be attributed to various factors, including geographical differences, breeding conditions and poultry farm environments (Sobur et al., 2019). Further, these differences could be attributed to factors such as flock size, lower immunity levels, and the specific type of poultry species investigated in each study.

During the current study, plasmid DNA was screened using PCR to detect sulfonamide-resistant genes (*sul-1, sul-2*, and *sul-3*). The PCR results revealed that 42.85% (9/21) of the strains harbored the sul-1 gene, while 38.09% (8/21) showed the presence of the *sul-2* gene. Additionally, 19.04% (4/21) of the strains coharbored both *sul-1* and *sul-2* genes. None of the isolates showed the presence of the *sul-3* gene. Furthermore, class 1 and class 2 integrons were detected in 80.95% (17/21) and 19.04% (4/21) of the strains, respectively. One of the previous studies in Pakistan has reported a high incidence rate of *sul-1* and *sul-2* genes which were associated with class 1 integrons (Kashif et al., 2013). This study also described the sulfonamide resistance even though the isolates were negative for the presence of *sul*-genes, suggesting the presence of other sulfonamide resistance genes (Kashif et al., 2013).

Similar kind of studies have been conducted in Egypt and Pakistan and reported the prevalence of 100 and 78% for the *sul-1* gene among *E. coli* strains, respectively (Shehata et al., 2016; Ahmad et al., 2018). Another investigation highlighted the widespread prevalence of class 1 integrons carrying sulfonamide resistance genes, including *sul-1* and *sul-2*, among chicken strains in Pakistan (Waseem et al., 2019). A comparable report described the dissemination of sulfonamide resistance genes, both individually and in combination, among livestock and poultry farms (Wang et al., 2014). Sulfonamide resistance genes have been consistently identified in chicken and other animal studies (Kozak et al., 2009a, b; McKinney et al., 2010), and these genes may be disseminated through various mechanisms. The *sul-1* gene is frequently found on integrons, while *sul-2* is commonly associated with plasmids (He et al., 2014). The presence of class 1 integrons is responsible for the diversity and transmission of multiple resistant strains in poultry industry (Moura et al., 2012).

Previously, it was described in Pakistan that significant burden of sulfonamide resistance genes was observed with 47% carrying *sul-1*, 60% carrying *sul-2*, 38% containing class 1 integrons, and 2% harboring class 2 integrons in poultry birds (Idrees et al., 2011). The results of the current study align with studies conducted in various countries, indicating that *E. coli* may carry integrons that facilitate the spread of resistance among food-producing animals (Song et al., 2010; Zulqarnain et al., 2021). Integrons could transmit resistance genes from commensal to pathogenic bacteria, and they can carry multiple resistance genes simultaneously (Van Essen-Zandbergen et al., 2009; Khan et al., 2021; Zulqarnain et al., 2021; Sagor et al., 2022). The detection of resistance genes and integrons in our study suggests a significant risk of antibiotic resistance dissemination, as gene cassettes can duplicate and rearrange within integrons, increasing the transmission of resistance genes among *E. coli* strains (O'Brien, 2002). Contact with animals or their excreta have been proposed as the main route for transmission of resistant pathogens from food-

producing animals to humans (Hassan Ali et al., 2010). It is also worth noting that food of animal origin can serve as a vehicle for the transmission of resistance genes and bacteria between humans and animals (van den Bogaard and Stobberingh, 2000).

Previous studies have confirmed that ST156 and ST23 are associated with *E. coli* strains found in humans (Corvec et al., 2010; Yamaji et al., 2018). The current study showed that ST155 was the second most reported sequence type among chicken birds, the results are similar with a study conducted in Egypt that demonstrated its association APEC and avian fecal *E. coli* (**AFEC**) (Hussein et al., 2013).

MLST analysis also revealed overlapping STs among different specimens. For example, ST48 and ST156 were reported in strains from both chicken and beef. ST48 corresponds to CC10, which is well-known for its association with diarrhea in humans, indicating that interspecies transmission of these clones may contribute to the overlapping of STs among different hosts (Yu et al., 2018). One of the studies conducted in China reported a high prevalence of ST746, ST156, ST350, and ST23 among sulfonamide-resistant *E. coli* strains of poultry origin which is similar to the findings of the current study (Li et al., 2022). A similar study from Nigeria reported multiple STs, including ST1638, ST155, and ST48, associated with strains from both chickens and humans, suggesting that the transfer of multiple resistance genes through plasmids may contribute to the emergence and distribution of STs among food-producing animals and humans (Aworh et al., 2021).

CONCLUSIONS

The emergence and spread of these specific *E. coli* strains (STs) underscore the diversity within the *E. coli* isolates pose a significant risk to both animals and humans. Further studies are needed to monitor these resistance determinants and to develop effective strategies to control antibiotic resistance to monitor its spread among humans.

In summary, our findings reveal the presence of various STs carrying the *sul-1* and *sul-2* genes among *E. coli* strains from commercial broilers. The results suggested that the diversity of *E. coli* strains may contribute to the development and dissemination of antibiotic resistance. Additionally, it is recommended to implement regulatory measures for monitoring sulfonamides and other antimicrobials in commercial poultry settings according to the prescribed antimicrobials.

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DISCLOSURES

No potential conflict of interest was reported by the authors.

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