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Proceeding Paper

Resistotyping of *Salmonella* spp. and *Staphylococcus aureus* from Milk and Milk Products Sold in Sabon-Gari and Zaria Local Government Areas of Kaduna State, Nigeria †

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Abstract: This study investigated the resistotyping of *Salmonella* spp. and Staphylococcus aureus from milk and milk products sold in Sabon-gari and Zaria Local Government Areas of Kaduna State, Nigeria. A total of 122 isolates; *Salmonella* spp. (65) and *Staphylococcus aureus* (57) were isolated from 400 milk and milk products. The isolates were subjected to antimicrobial susceptibility testing using the disc diffusion and E-test methods. The results obtained from the study indicated that 39 (31.967%) isolates were sensitive to all tested antibiotics, while 47 (38.525%) were resistant to a single antibiotic. 36 (29.508%) were resistant to two antibiotics, and none showed resistance to at least three antibiotics. None showed resistance to all four antibiotics. Resistance rates were most frequently observed in Tetracycline at 80 (65.574%), followed by Ampicillin at 39 (31.967%), Gentamicin, and Ciprofloxacin both at 00 (00.000%). After comparing with the CLSI and EUCAST breakpoints, the resistance rate with CLSI was Tetracycline at 104 (85.245%), followed by Ampicillin at 39 (66.393%), Ciprofloxacin at 14 (11.475%), and Gentamicin at 06 (04.918%). The resistance rate with EUCAST was Tetracycline at 122 (100.000%), followed by Ampicillin at 110 (90.164%), Ciprofloxacin at 88 (72.131%), and Gentamicin at 17 (13.934%). Based on these findings, it has been shown that *Salmonella* spp. and *Staphylococcus aureus* found in milk and milk products within Sabon-gari, and Zaria Local Government Areas have a high resistance to the antibiotics tested. It is imperative that urgent actions are taken to address the growing menace of AMR and prevent the spread of antibiotic-resistant pathogens.

Keywords: *Salmonella* spp.; *Staphylococcus aureus*; Ampicillin; Ciprofloxacin; Gentamicin; Tetracycline

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1. Introduction

Milk is the fluid secreted by female mammals for nourishing offspring. It comprises a mixture of complex chemical substances which include fat, protein, lactose, and some mineral matters in the colloidal state in the form of a true solution [1].

Resistotyping involves grouping bacterial isolates based on resistance patterns to a set of randomly chosen antibiotics peculiar to specific strains by phenotypic methods.

Antimicrobial resistance (AMR) is on the increase and has posed a major public health concern, severely limiting therapeutic options in clinical settings [2].

2. Materials and Methods

2.1. Study Area

The study area included two (2) local government areas (Soban-gari and Zaria) in Kaduna state, see Figure 1.

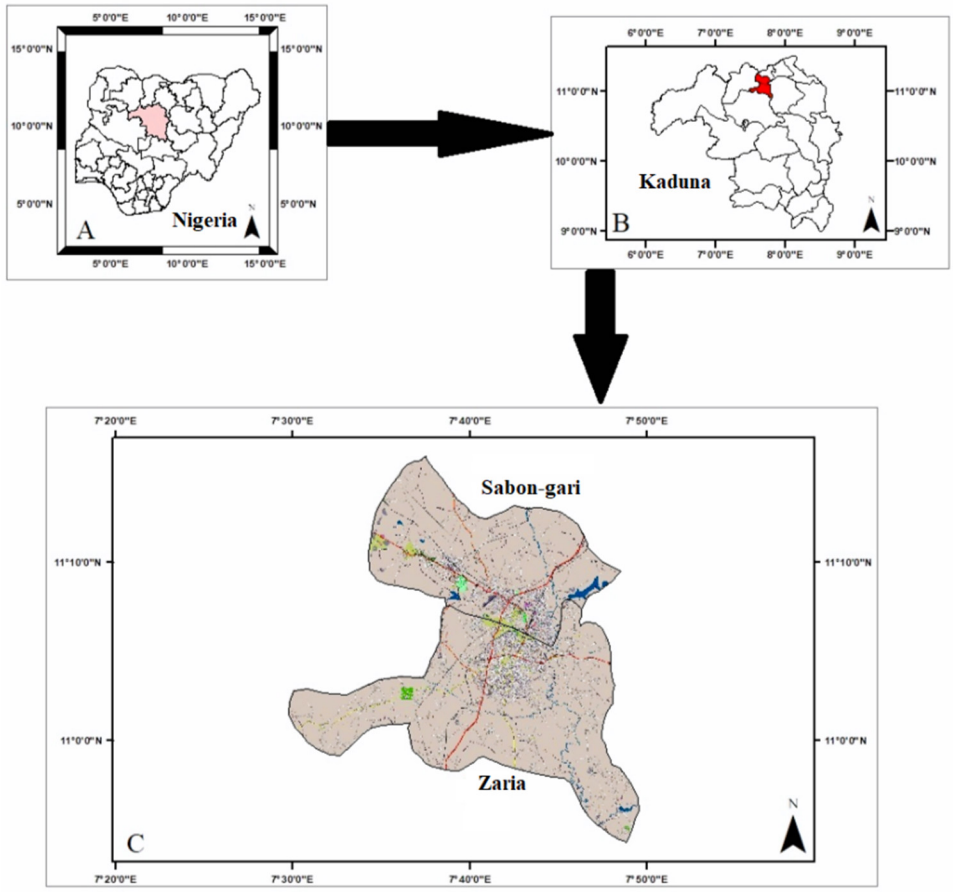


Figure 1. Map of Zaria and Sabon-gari.

2.2. Isolation of organisms

Four hundred (400) samples [100 each] of milk and milk products (Kindirimo, Nono, and Yogurt) sold in Zaria and Sabon-gari.

2.2.1. Pre-Enrichment

Twenty-five (25) grams of the sample were weighed and poured into 225 mL of Buffered peptone water, and incubated at 37 °C for 24 h.

2.2.2. Selective Plating for *Staphylococcus aureus*

After incubation, a loopful of inoculum (from above) was inoculated on the Mannitol Salt Agar (MSA) plate and incubated at 37 ± 1 °C for 30 h. and observed for growth (yellow halo) indicates *Staphylococcus (S.) aureus*.

2.2.3. Selective Plating for *Salmonella* spp.

To isolate *Salmonella* spp., one (1) ml of pre-enrichment culture was inoculated into Rappaport-Vassiliadis Broth (RV) and incubated at 42 °C for 7 days. After the incubation,

it was further cultured on selective agar plates of *Salmonella-Shigella* Agar (SSA) at 37 °C for 48 h.

2.3. Antimicrobial Susceptibility Test (AST)/Resistotyping

The AST for Ampicillin (AMP), Ciprofloxacin (CIP), Gentamicin (GEN), and Tetracycline (TET) were performed concurrently with Disc Diffusion Test (Bioanalyse, Turkey) using AM30g, CIP05g, GM10g, and TE10g concentrations, respectively, and E-Test (HiMedia, India) using 0.016-256 g/mL concentrations [3]. Mueller-Hinton Agar plates (HiMedia, India) were made fresh for each test. The plates were inoculated with standardized inoculum (0.5 McFarland Standard) of isolates and incubated at 37 °C for 24 h. The Minimum Inhibitory Concentration (MIC) and Zone of Inhibition (ZOI) values of AMP, CIP, GEN, and TET are interpreted as S (Susceptible), I (Intermediate), or R (Resistant) based on the breakpoints indicated by the reference standards for Clinical and Laboratory Standards Institute (CLSI) on http://em100.edaptivedocs.net [4] and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) on http://www.eucast.org [5].

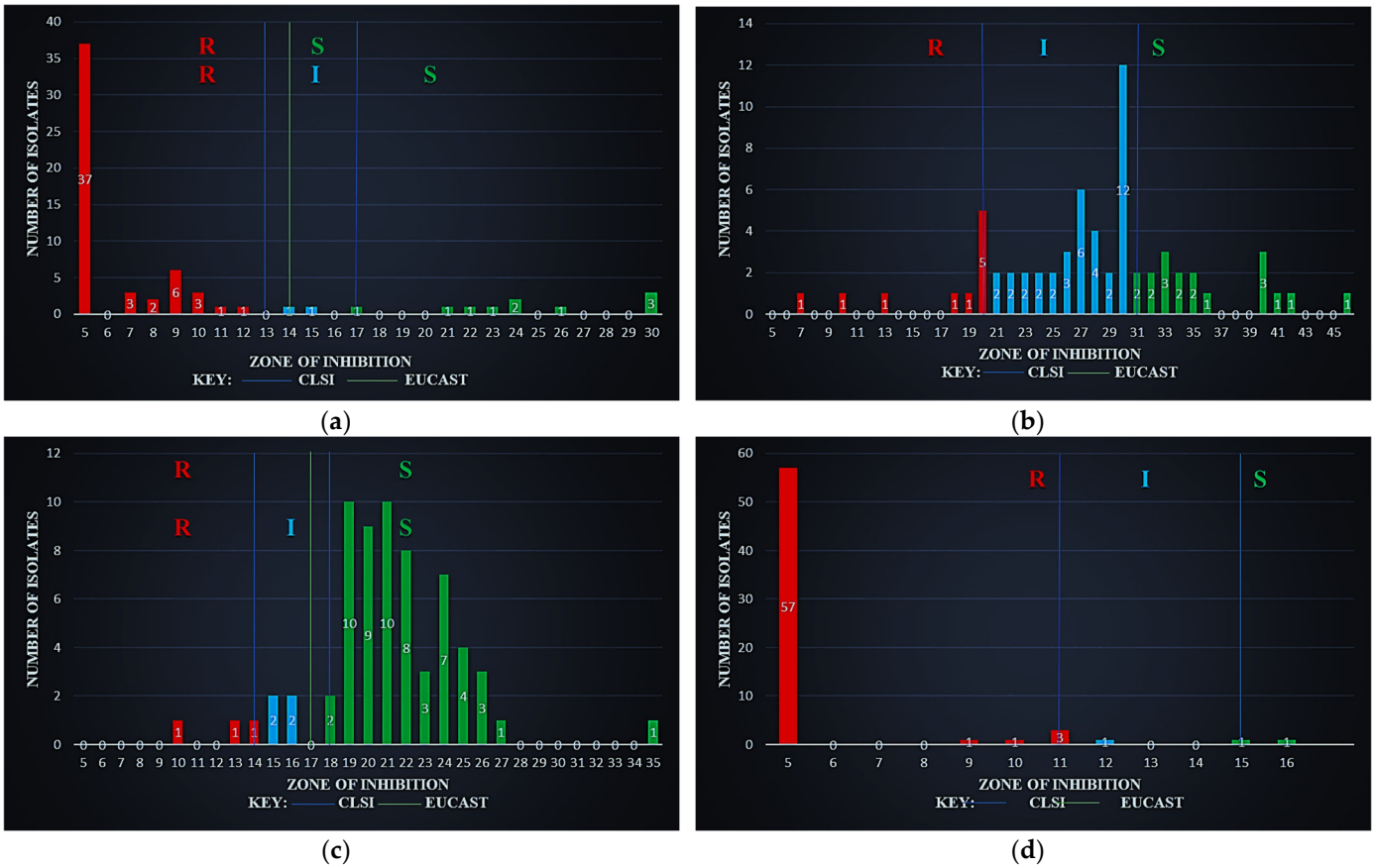
3. Results

3.1. Organisms Isolated

A total of 122 isolates; *Salmonella* spp. (65) and *S. aureus* (57) were isolated from the samples.

3.2. The ZOI Results

Figure 2a shows the ZOI for the antibiotic AMP against *Salmonella* spp. using the CLSI (R ≤ 13, I 14-16, S ≥ 17) and EUCAST (R < 14, S ≥ 14) breakpoints. CLSI showed that 53 (81.538%) of the isolates were R, while 02 (03.077%) were I and 10 (15.385%) whereas EUCAST indicated 53 (81.538%) R and 12 (18.462%) S.



(**a**) (**b**)

(**c**) (**d**)

Figure 2. Zone of Inhibition against *Salmonella* spp.: (**a**) Using Ampicillin (AM30µg). (**b**) Using Ciprofloxacin (CIP05µg). (**c**) Using Gentamicin (GM10µg). (**d**) using Tetracycline (TE10µg).

Figure 2b shows the CLSI breakpoints for ZOI (CIP) on *Salmonella* spp. are R ≤ 20, I 21-30, and S ≥ 31; however, EUCAST had no breakpoints available. Based on CLSI, 10 (15.385%) were R, 37 (56.923%) I, and 18 (27.692%) S.

Figure 2c shows the ZOI (GEN) on *Salmonella* spp. with CLSI breakpoints R ≤ 14, I 15-17, S ≥ 18; about 03 (04.615%) R, with 04 (06.154%) I and 58 (89.231%) S, whereas on EUCAST (R < 17, S ≥ 17), 07 (10.769%) R and 58 (89.231) S.

Figure 2d shows the ZOI (TET) with a CLSI breakpoint of R ≤ 11, I 12-14, S ≥ 15. There were no EUCAST breakpoints for the tetracycline. However, the CLSI gave 62 R (95.385%), 01 I (01.538%), and 02 S (03.077 %).

Figure 3a shows the ZOI results for the antibiotic AMP against *S. aureus* with the CLSI breakpoints (R < 22, S ≥ 22), moreover, the EUCAST has no breakpoints. Based on CLSI, 30 (52.632%) of the isolates were R, and 27 (47.368%) S.

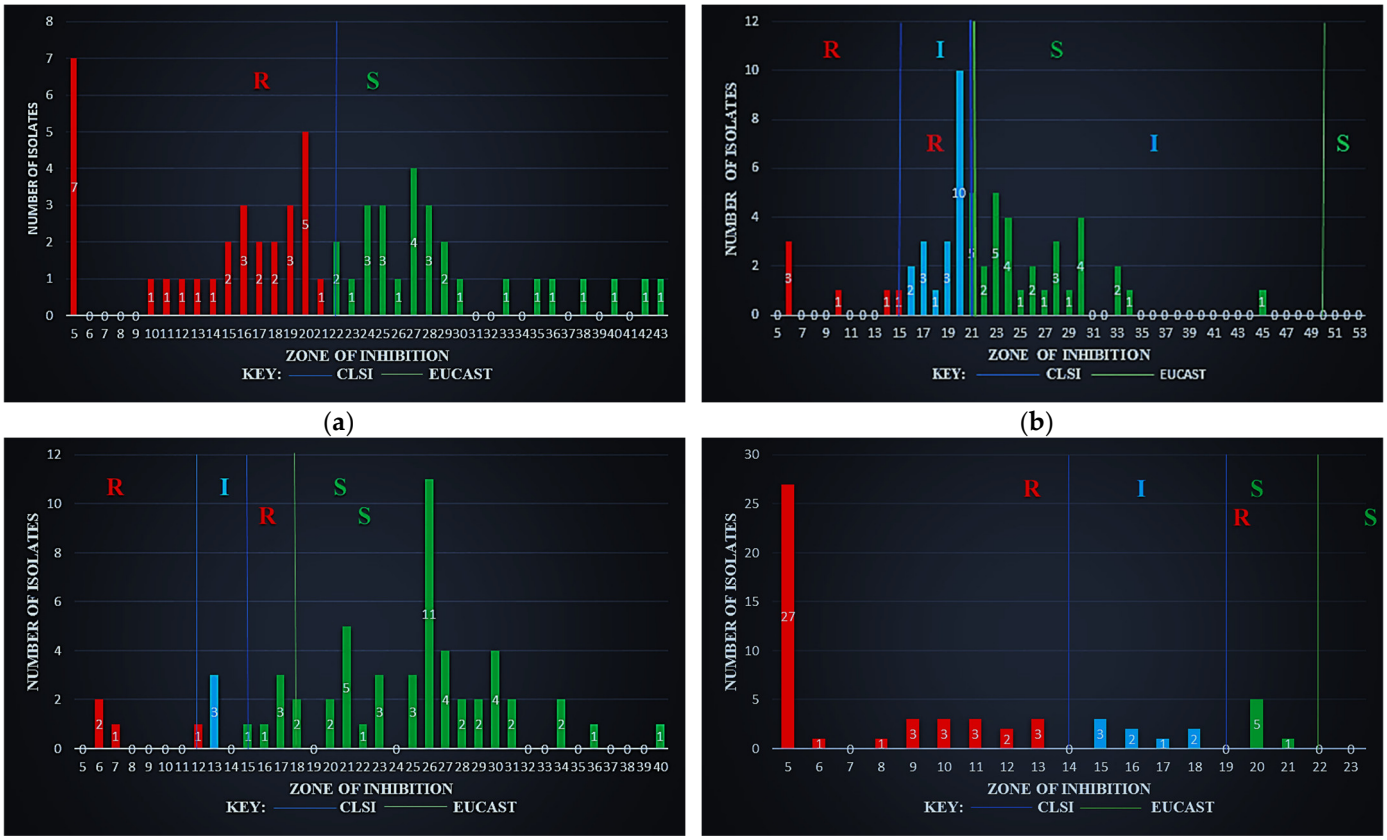


Figure 3. Zone of Inhibition against *S. aureus*: (**a**) Using Ampicillin (AM30µg). (**b**) Using Ciprofloxacin (CIP05µg). (**c**) Using Gentamicin (GM10µg). (**d**) using Tetracycline (TE10µg)

Figure 3b shows the ZOI results for the antibiotic CIP against *S. aureus* with the CLSI breakpoints (R ≤ 15, I 16-20 S ≥ 21), and EUCAST breakpoints (R ≤ 21, I 22-49 S ≥ 50). Based on CLSI, 06 (10.526%) of the isolates were R, with 19 (33.333%) I and 32 (56.140%) S, whereas, on EUCAST, 25 (43.860%) R, 32 (56.140%) I, and 00 (00.000%) S.

Figure 3c shows the ZOI results for the antibiotic GEN against *S. aureus* with the CLSI breakpoints (R ≤ 12, I 13-14 S ≥ 15), and EUCAST breakpoints (R < 18, S ≥ 18). Based on CLSI, 04 (07.018%) of the isolates were R, with 03 (05.263%) I and 50 (87.719%) S, whereas, on EUCAST, 14 (24.561%) R, 43 (75.439%) S.

Figure 3d shows the ZOI results for the antibiotic TET against *S. aureus* with the CLSI breakpoints (R ≤ 14, I 15-18 S ≥ 19), and EUCAST breakpoints (R < 22, S ≥ 22). Based on

CLSI, 43 (75.439%) of the isolates were R, with 08 (14.035%) I and 06 (10.526%) S, whereas, on EUCAST, 57 (100.000%) R, with S 00 (00.000%).

3.3. AST Summary

The data presented in Table 1 shows that *Salmonella* spp. and *S. aureus* displayed 4 distinct Antimicrobial Resistance Patterns (ARPs) across Groups I, L, O, and P. The largest R population of 44 (36.066%) was in Group O with primary resistance to TET. Then Group P (31.967%) in which all are S. Group I (29.508%) where AMP and TET were R and Group L with the least 02.459% where only AMP was R. From these four (4) groups, representatives were selected for the E-test MIC i.e., 2 per group, total of 16 organisms.

Table 1. AST Summary Based on ZOI.

Group	Antimicrobial Resistance Pattern (Resistotyping)	Number of Antibiotics	MAR Index	Number of Isolates	Percent	Organism (Percent)
A	AMP-CIP-GEN-TET	4	1.00	000	00.000	–
B	AMP-CIP-GEN-TET	3	0.75	000	00.000	–
C	AMP-CIP-GEN-TET	3	0.75	000	00.000	–
D	AMP-CIP-GEN-TET	3	0.75	000	00.000	–
E	AMP-CIP-GEN-TET	3	0.75	000	00.000	–
F	AMP-CIP-GEN-TET	2	0.50	000	00.000	–
G	AMP-CIP-GEN-TET	2	0.50	000	00.000	–
H	AMP-CIP-GEN-TET	2	0.50	000	00.000	–
I	AMP-CIP-GEN-TET	2	0.50	036	29.508	*Salmonella* spp. (91.7) *S. aureus* (08.3)
J	AMP-CIP-GEN-TET	2	0.50	000	00.000	–
K	AMP-CIP-GEN-TET	1	0.50	000	00.000	–
L	AMP-CIP-GEN-TET	1	0.25	003	02.459	*Salmonella* spp. (33.3) *S. aureus* (66.7)
M	AMP-CIP-GEN-TET	1	0.25	000	00.000	–
N	AMP-CIP-GEN-TET	1	0.25	000	00.000	–
O	AMP-CIP-GEN-TET	1	0.25	044	36.066	*Salmonella* spp. (50) *S. aureus* (50)
P	AMP-CIP-GEN-TET	0	0.00	039	31.967	*Salmonella* spp. (23.1) *S. aureus* (76.9)
Total	083-122-122-042			122	100.000	

Key: Green—Susceptible (≥ 6 mm), Red—Resistance (=5 mm).

3.4. E-Test Summary

Table 2 highlights the E-Test pattern. The MIC breakpoints for *Salmonella* spp., CLSI (AMP S ≤ 08 I16 R ≥ 32; CIP S ≤ 0.25 I0.5 R ≥ 01; GEN S ≤ 02 I04 R ≥ 08, TET S ≤ 04 I08 R ≥ 16). A variation was specifically observed for *Salmonella* spp., selected isolates from Groups I and L were S to AMP despite being earlier R, the selected organisms that were earlier CIP S were now R and TET resistance was. While for *S. aureus*, CLSI (AMP S ≤ 04 I– R ≥ 08, CIP S ≤ 01 I02 R ≥ 04, GEN S ≤ 04 I08 R ≥ 16, TET S ≤ 04 I08 R ≥ 16). A variation was also specifically observed for *S. aureus*, selected isolates from Groups I, and L which were S to AMP are now R, while Group O now showed S when it should be R to only TET.

Table 2. E-Test Summary.

Organism	Antibiotic Strip (Abb.)	Concentration [μg/mL]	CLSI Breakpoints (MIC) [μg/mL]			Number of Isolates *		
			S	I	R	S	I	R
Salmonella spp.	Ampicillin (AMP)	0.016–256	≤08	16	≥32	06	–	02
	Ciprofloxacin (CIP)	0.016–256	≤0.06	0.12–0.5	≥01	02	02	04
	Gentamicin (GEN)	0.016–256	≤02	04	≥08	08	–	–
	Tetracycline (TET)	0.016–256	≤04	08	≥16	03	02	03
Staphylococcus aureus	Ampicillin (AMP)	0.016–256	≤04	–	≥08	04	–	04
	Ciprofloxacin (CIP)	0.016–256	≤01	02	≥04	06	02	02
	Gentamicin (GEN)	0.016–256	≤04	08	≥16	05	01	01
	Tetracycline (TET)	0.016–256	≤04	08	≥16	03	02	03

4. Discussion

The study conducted by Tamba et al., 2016 [6] on *Salmonella* isolates showed resistance rates AMP (85.7%), TET (35.7%), CIP (00.0%), and GEN (00.0%). Their findings indicated that AMP is the most resistant drug. Our study shows there is an increase in resistance rates among *Salmonella* isolates on all the other drugs tested, at TET had (95.385%) on CLSI breakpoint only, CIP (15.385%), and GEN (04.615%) respectively on both CLSI and EUCAST. While AMP showed a drop from 85.7% to 81.538%, TET had an alarming jump from 35.7% to 95.385%.

In the case of *S. aureus* isolates, Umaru et al., 2013 [7] used the CIP, GEN, and TET along with others, the resistance rate was TET (55.5%), CIP (38.9%), GEN (11.1%), and oxacillin (100.0%) which can be substituted for AMP [4], thus, making AMP, the most resistance drug in that study. This study shows there is an increase in resistance rates among *S. aureus* isolates on all the drugs tested; TET on the CLSI (75.439%), and on EUCAST (100.000%), AMP (52.632%) CLSI only, CIP on the CLSI (10.526%), on EUCAST (43.860%), and GEN on CLSI (10.526%) and on EUCAST (43.860%). The same pattern is observed here an alarming jump in TET (55.5% to 75.439%) and a drop in AMP (100.0% to 52.632%).

From Table 1, half of the selected isolates are resistant to AMP and TET, with all being susceptible to CIP AND GEN. However, in Table 2, AMP in *Salmonella* spp. was 25%R, and *S. aureus* was 50%R, TET was 37.5%R for both, CIP was 50%R and 25%R respectively, GEN was 00%R and 12.5% R respectively.

In summary, recent research has revealed a surge in antibiotic resistance, particularly in TET, which is the most resistant drug for both organisms. AMP, CIP, and GEN follow in that sequence. Prompt measures must be taken to tackle the escalating issue of antimicrobial resistance (AMR) and curb the proliferation of antibiotic-resistant pathogens.

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