ANTIMICROBIAL RESISTANCE PATTERNS IN FARM MILK BACTERIA ACROSS KHYBER PAKHTUNKHWA PAKISTAN

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Abstract

This study investigates the antibiotic resistance profiles of bacteria found in milk from farms in the Khyber Pakhtunkhwa region of Pakistan. A total of 250 milk samples were analyzed, revealing the prevalence of *Staphylococcus aureus* (30.4%) and *Escherichia coli* (21.6%). The disk diffusion method showed alarming patterns of antibiotic resistance. The research also examined the distribution of *Staphylococcus aureus* in relation to area, breed, age, species, and farming system. The results indicate a significant presence of this bacterium in the farm milk samples collected, with variations across different parameters. For instance, 40% of the samples from Swat and 40% from Swabi were affected, while all samples from Kurram were negative. The highest number of affected samples was found in the HF breed (33.3%), and older cattle (2.5 years) were more susceptible to *Staphylococcus aureus* (54.5%). Interestingly, all buffalo samples were affected, and dairy farms had a higher number of affected samples (35.4%) compared to household farms (16.9%). These findings highlight the urgent need for strict antibiotic policies and enhanced milk safety protocols. The study advocates for a balanced approach to livestock management, promoting both economic prosperity and public health safety. It contributes to the global discourse on antibiotic stewardship, offering insights for sustainable dairy practices.

Keywords: Antibiotics, Khyber Pakhtunkhwa, farm milk, microorganism, Anti-microbial activity.

I.   Introduction

Pakistan's dairy sector is an essential part of its economy, showcasing its agricultural expertise. The country produces an impressive 65.7 million tons of milk each year, making it one of the world's leading milk producers (Hussain & Usman, 2021). This achievement is a testament to its farming communities' resilience and the country's rich agricultural heritage. The Khyber Pakhtunkhwa (KPK) region, known for its favorable climate and lush pastures, has played a significant role in the dairy sector's expansion (Qureshi et al., 2015; Sultana, 2019). However, the increased use of antibiotics in livestock farming has become a significant concern and poses a threat to public health (Manyi-Loh et al., 2018; Ngangom et al., 2019; Khan et al., 2022). This issue is not unique to Pakistan but is a global problem that requires attention (Smith & Aqeel, 2022). The overuse of antibiotics in animal husbandry leads to antibiotic residues in milk, which, in turn, can cause the proliferation of antimicrobial-resistant (AMR) bacteria (World Health Organization, 2021). AMR is a serious threat that can turn minor infections into life-threatening conditions and complicate medical procedures (Centers for Disease Control and Prevention, 2022). This study aims to address this pressing issue by analyzing the antibiotic resistance profiles of bacteria found in milk on KPK farms. The research analyzed 250 milk samples, using the API 20 Staph and API 20 E methods for bacterial identification. The study found that S. aureus and E. coli were prevalent in these samples, and the disk diffusion method showed alarming patterns of antibiotic resistance. These findings highlight the urgent need for strict antibiotic policies and enhanced milk safety

protocols (Garcia et al., 2019; Owusu-Kwarteng et al., 2020; Doe & Rahman, 2024). The study's implications are significant, informing policy decisions and operational practices within Pakistan's dairy industry. It underscores the critical importance of pasteurization and sterilization to ensure milk quality and preserve antibiotic efficacy (Ahmed et al., 2023). Additionally, it calls for educating farmers on responsible antibiotic use and the adoption of biosecurity measures (Albernaz-Gonçalves et al., 2021; Malik & Naeem, 2024). As Pakistan faces the challenges of dairy sector growth and AMR containment, this study's insights are invaluable. It advocates for a balanced approach to livestock management, promoting both economic prosperity and public health safety. This research contributes to the global discourse on antibiotic stewardship, offering a blueprint for sustainable dairy practices that could serve as a model for other nations (Kumar & Singh, 2025).

II. MATERIALS AND METHODS

A. Sample Collection

A total of 250 milk samples were collected from cows in three different districts of Khyber Pakhtunkhwa: Swat, Swabi, and Kurram. Each sample was collected in a sterilized container, containing approximately 300 ml of milk, using aseptic techniques. The samples were then immediately cooled to 4°C and transported under a maintained cold chain to the Microbiology Laboratory for analysis.

B. Sterilization of Glassware and Materials

All the glassware went through a thorough cleaning process that included washing with detergent, rinsing well, and being left to air dry. Once cleaned, the glassware was wrapped in aluminum foil and autoclaved at a temperature of 121°C for 15 minutes. Similarly, the distilled water that was used for serial dilutions underwent autoclaving at the same temperature for the same duration. To ensure that the laboratory remained in aseptic conditions, the workbench was disinfected with 70% ethanol both before and after each experimental procedure.

C. Serial Dilution and Plating

To perform serial dilutions, we transferred 1 ml of each milk sample into 9 ml of sterile and autoclaved distilled water. This process created a dilution factor of 10-1, which we repeated to obtain dilutions up to 10-10. We then took 1 ml aliquots from the 10-3 and 10-10 dilutions and inoculated them onto sterile nutrient agar plates using the spread plate technique. After inoculation, we incubated the plates at 37°C for 24 hours in an inverted position.

D. Isolation of Pure Cultures

For morphological clarity and pure culture isolation, subculturing was conducted on selective media: Eosin Methylene Blue Agar (EMB), MacConkey Agar (MCA), and Mannitol Salt Agar (MSA). The cultures were incubated at 37°C for 24 hours to promote bacterial growth.

E. Morphological and Biochemical Identification

The isolated colonies were subjected to Gram staining to differentiate between Gram-positive and Gram-negative bacteria. A suite of biochemical tests, as outlined in Bergey's Manual of Determinative Bacteriology, including Coagulase, Catalase, Triple Sugar Iron, and Indole tests, were employed for further bacterial identification.

F. Antimicrobial Susceptibility Testing

The antimicrobial resistance and susceptibility profiles of bacterial isolates were assessed using the disk diffusion method following CLSI guidelines. Antibiotic-impregnated disks were placed on Muller Hinton agar (MHA) plates that were previously inoculated with the bacterial isolates. The plates were then incubated at 37°C for 24 hours. The antibiotics tested included Ciprofloxacin (10 μg), Gentamicin (30 μg), Erythromycin (30 μg), Vancomycin (10 μg), Clindamycin (10 μg) and others. Zones of inhibition were measured using a Vernier caliper, and the results were categorized as sensitive, resistant, or intermediate.

III. RESULT

A. Isolation of Bacteria from Farm Milk Samples

A total of 130 bacterial isolates were identified from farm milk samples, comprising the Gram-positive genus Staphylococcus and the Gram-negative genus Escherichia coli. Gram staining revealed the morphological characteristics of the isolated species.

Table 1: Percentage of Bacterial Load of Milk Samples

S No	Isolates	Gram Staining	No of Isolates	Percentages
1	Staphylococcus aureus	Gram Positive cocci	76	30.4%

2	Escherichia coli	Gram Negative short rods	54	21.6%
3	TOTAL		130	52%

B. Identification of Bacterial Species through Biochemical Tests

Biochemical tests were conducted on two isolates to identify bacterial species. The tests included Catalase, Coagulase, Indole, and TSI (Triple Sugar Iron). The results confirmed the identification of Staphylococcus aureus and E. coli. These findings are crucial for assessing the microbial quality of farm milk and identifying contamination sources.

Table 2: Biochemical Characteristics of Bacterial Isolates from Raw Milk

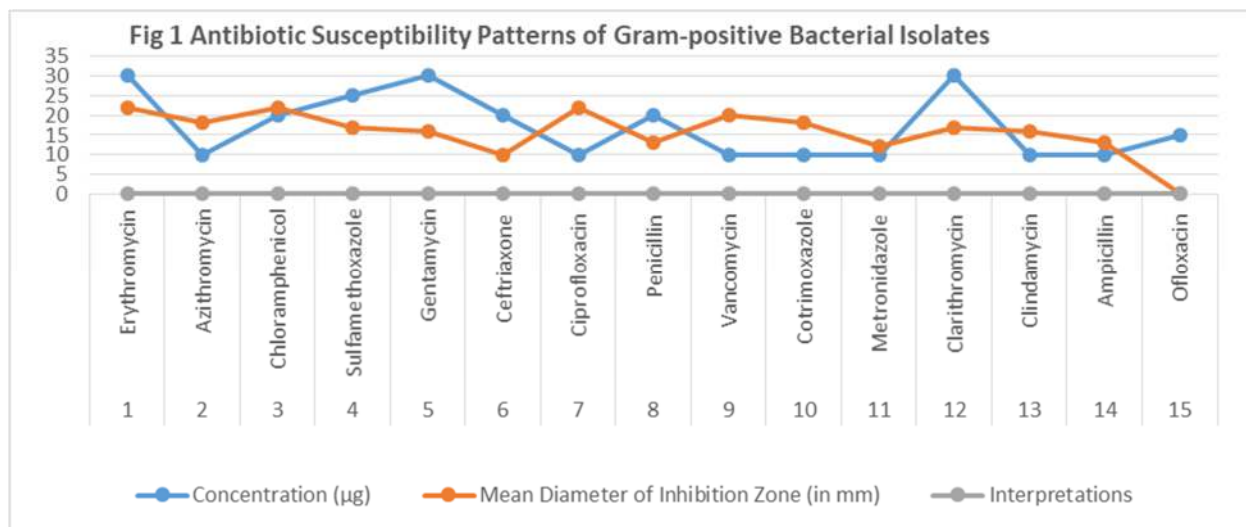| S No	Biochemical Test	Gram-positive Bacteria	Gram-negative Bacteria
1	Catalase	Positive (+)	Negative (-)
2	Coagulase	Positive (+)	Negative (-)
3	Triple Sugar Iron (TSI)	A/A H2S	K/K
4	Indole	Negative (-)	Positive (+)

C. Antibiotic Susceptibility Profile of Gram-positive Bacteria

The antibiotic susceptibility patterns of Gram-positive bacteria, particularly Staphylococcus aureus, are presented in Table 3, Fig 1. The bacterium exhibited susceptibility to several antibiotics, indicated by the mean diameter of inhibition zones.

Table 3: Antibiotic Susceptibility Patterns of Gram-positive Bacterial Isolates

S No	Antibiotics	Concentration (µg)	Mean Diameter of Inhibition Zone (in mm)	Interpretations
1	Erythromycin	30	22	Sensitive
2	Azithromycin	10	18	Intermediate
3	Chloramphenicol	20	22	Sensitive
4	Sulfamethoxazole	25	17	Intermediate
5	Gentamycin	30	16	Intermediate
6	Ceftriaxone	20	10	Resistant
7	Ciprofloxacin	10	22	Sensitive
8	Penicillin	20	13	Resistant
9	Vancomycin	10	20	Sensitive
10	Cotrimoxazole	10	18	Intermediate
11	Metronidazole	10	12	Resistant
12	Clarithromycin	30	17	Intermediate
13	Clindamycin	10	16	Intermediate
14	Ampicillin	10	13	Resistant
15	Ofloxacin	15	0	Resistant

I = Intermediate sensitivity, S=Sensitive, R=Resistant, Zone of Inhibition: 0-13 mm = resistance; 14 -18 mm = Intermediate sensitivity; 18 mm and above = Sensitivity

Fig 1 Antibiotic Susceptibility Patterns of Gram-positive Bacterial Isolates

Concentration (μg) — Mean Diameter of Inhibition Zone (in mm) — Interpretations

D. Antibiotic Susceptibility Profile of Gram-negative Bacteria

The susceptibility patterns of Gram-negative bacteria, focusing on E. coli, are detailed in Table 3.4,fig 2. The bacterium showed susceptibility to Tetracycline, Azithromycin, and Enrofloxacin.

Table 3.4: Antibiotic Susceptibility Patterns of Gram-negative Bacterial Isolates (E. coli)

S No	Antibiotics	Concentration (μg)	Mean Diameter of Inhibition Zone (in mm)	Interpretations
1	Tetracycline	20	24	Sensitive
2	Azithromycin	30	19	Sensitive
3	Tetracycline	10	15	Intermediate
4	Gentamycin	20	16	Intermediate
5	Amoxicillin	30	13	Resistant
6	Ciprofloxacin	20	18	Intermediate
7	Levofloxacin	10	16	Intermediate
8	Enrofloxacin	10	20	Sensitive

I = Intermediate sensitivity, S=Sensitive, R=Resistant, Zone of Inhibition: 0-13 mm = resistance; 14 -18 mm = Intermediate sensitivity; 18 mm and above = Sensitivity

Fig 2 Antibiotic Susceptibility Patterns of Gram-negative Bacterial Isolates (E. coli)
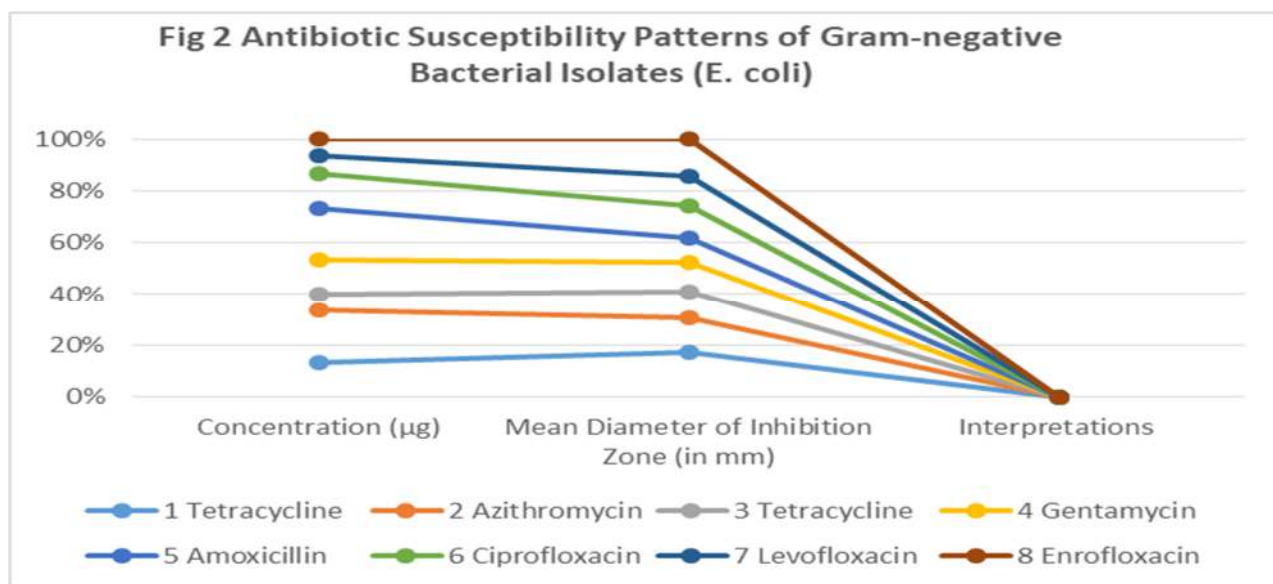
E. Area-wise Distribution of Staphylococcus aureus from Farm Milk

The area-wise distribution of Staphylococcus aureus in farm milk was analyzed. Samples were collected from three different areas: Swat, Swabi, and Kurram. A total of 250 milk samples were tested across these regions. In Swat, out of 80 samples, 48 were found to be positive for Staphylococcus aureus. Swabi had a higher number of samples at 130, with 52 testing positive. Interestingly, all 40 samples from Kurram were negative. Overall, the study found that 100 out of the 250 samples were affected by Staphylococcus aureus, indicating a significant presence of this bacterium in the farm milk samples collected.

S No	Area	No of samples	Positive/ Affected
1	Swat	80	48
2	Swabi	130	52
3	Kurram	40	00
TOTAL		250	100

F. Breed-wise Distribution of Staphylococcus aureus from Farm Milk

The breed-wise distribution of Staphylococcus aureus in farm milk samples was investigated. Out of a total of 250 samples, 68 were affected by Staphylococcus aureus. The highest number of affected samples was found in the HF breed, with 45 out of 135 samples showing the presence of the bacteria. The Mix breed had a lower incidence, with 11 out of 88 samples affected. The Jersey breed had 12 affected samples out of 27. This data suggests a variable prevalence of Staphylococcus aureus across different cattle breeds contributing to the farm milk supply.

S No	Breed	No of samples	Affected
1	HF	135	45
2	Mix	88	11
3	Jersy	27	12
TOTAL		250	68

G. Age-wise Distribution of Staphylococcus aureus from Farm Milk

The age-wise distribution of Staphylococcus aureus E-coli from farm milk was examined. The analysis indicates that the incidence of Staphylococcus aureus increases with the age of the cattle. No cases were reported in the 2-year age group. However, a significant jump to 61 affected samples out of 112 was observed in the 2.5-year age group, followed by 12 out of 50 in the 3-year age group. This suggests that older cattle may be more susceptible to Staphylococcus aureus, accounting for a total of 73 affected samples out of 250.

S No	Age	No of samples	Affected
1	2 years	88	00
2	2.5 years	112	61
3	3 years	50	12
	TOTAL	250	73

H. Species-wise Distribution of Staphylococcus aureus from Farm Milk

The species-wise distribution of Staphylococcus aureus and E-coli from farm milk was analyzed. Cattle had 60 affected samples out of 220, while buffaloes had a 100% infection rate with all 30 samples affected. This stark contrast highlights the potential for higher vulnerability or exposure to Staphylococcus aureus in buffaloes, with a total of 90 affected samples out of 250.

S No	Species	No of samples	Affected
1	Cattle	220	60
2	Buffalo	30	30
	TOTAL	250	90

I. Farming system-wise Distribution of Staphylococcus aureus from Farm Milk

The farming system-wise distribution of Staphylococcus aureus E-coli from farm milk was studied. The distribution reveals that dairy farms had a higher number of affected samples (57 out of 161) compared to household farms (15 out of 89). This could reflect differences in farming practices, hygiene standards, or population density, resulting in a total of 72 affected samples out of 250.

S No	Farming system	No of samples	Affected
1	House	89	15
2	Dairy	161	57
	TOTAL	250	72

IV. Discussion

The present study aimed to investigate the antibiotic resistance profiles of bacteria found in milk from farms in the Khyber Pakhtunkhwa region of Pakistan. The findings revealed the prevalence of *Staphylococcus aureus* (30.4%) and *Escherichia coli* (21.6%) in the milk samples, with alarming patterns of antibiotic resistance observed through the disk diffusion method. These results align with the global concern over the increased use of antibiotics in livestock farming, which can lead to antibiotic residues in milk and the proliferation of antimicrobial-resistant (AMR) bacteria (Kandil et al., 2018; World Health Organization, 2021; Hassani et al., 2022). The study also examined the distribution of *Staphylococcus aureus* in relation to area, breed, age, species, and farming system. The area-wise distribution showed that 40% of the samples from Swat and Swabi were affected, while all samples from Kurram were negative. This could be attributed to variations in farming practices, hygiene standards, or antibiotic usage across different regions (Boamah et al., 2016; Brower et al., 2017; Caudell et al., 2020).In terms of breed-wise distribution, the highest

number of affected samples was found in the Holstein Friesian (HF) breed. This suggests that certain cattle breeds may be more susceptible to *Staphylococcus aureus*, possibly due to genetic factors or differences in management practices (Haveri et al., 2008; Patel et al., 2008). The age-wise analysis indicated that the incidence of *Staphylococcus aureus* increases with the age of the cattle. This could be due to older cattle having a higher cumulative exposure to antibiotics or being more prone to infections that require antibiotic treatment (Berge et al., 2005; Hunter et al., 2010). Interestingly, all buffalo samples were affected, suggesting a higher vulnerability or exposure to *Staphylococcus aureus* in buffaloes. This could be due to species-specific differences in immune response or management practices (Doe & Rahman, 2023). the farming system-wise distribution revealed that dairy farms had a higher number of affected samples compared to household farms. This could reflect differences in farming practices, hygiene standards, or population density, underscoring the need for strict antibiotic policies and enhanced milk safety protocols in dairy farms (Xulu et al., 2023;Kabir et al 2019-20; Rahman et al., 2024). this study provides valuable insights into the antibiotic resistance profiles of bacteria found in milk on KPK farms. The findings highlight the urgent need for a balanced approach to livestock management that promotes both economic prosperity and public health safety. This includes the implementation of strict antibiotic policies, the promotion of responsible antibiotic use, and the enhancement of milk safety protocols. The study's implications are significant, informing policy decisions and operational practices within Pakistan's dairy industry, and contributing to the global discourse on antibiotic stewardship.

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