

Isolation And Identification of Pathogenic Bacteria from Dairy Farm Animals and Determination of Their Antibigram

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ABSTRACT

Dairy farm animals are integral to milk production and agricultural economies, yet their health is often compromised by pathogenic bacteria. Therefore, this study aimed to assess the prevalence of pathogenic bacteria from dairy farm in Agriculture University, Peshawar kpk, using milk, fecal and water samples. A total of 25 samples (10 milk, 10 fecal and 5 water samples) were collected from a dairy farm and processed in the Microbiology Laboratory of the Institute of Allied Health Sciences, Sarhad University of Science and Information Technology, Peshawar. The samples were cultured on selective media, including Mannitol Salt Agar, MacConkey Agar and Shigella Agar. Morphological, gram staining and biochemical tests were performed to identify the bacterial isolates. The results revealed that *Escherichia coli* was the most prevalent pathogen (48 %), followed by *Salmonella* spp. (28 %) and *Staphylococcus* spp. (24 %). Antibiotic susceptibility testing showed that 83.33 % of *S. aureus* isolates were methicillin-resistant (MRSA), with Vancomycin and Linezolid being effective against all *S. aureus* isolates. All the gram-negative bacteria, which comprised 76 % of the isolates were multidrug resistant. Piperacillin/Tazobactam emerged as the most effective antibiotic for gram-negative isolates. In conclusion, the study showed

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significant bacterial contamination in dairy farm environments and underscores the need for effective antibiotic stewardship to combat resistance.

Keywords—Dairy farm animals, Linezolid, MRSA, Salmonella, *S. aureus*, Vancomycin

Introduction

Dairy animals have always been one of the most studied animal species in terms of animal welfare. Despite significant advancements in animal welfare practices, challenges remain, particularly concerning the well-being of dairy animals (Buller *et al.*, 2020). One major welfare issue is early weaning, a common practice where calves are separated from their mothers shortly after birth and fed artificial milk replacers. This practice, although designed to optimize milk production and streamline farm operations, has raised substantial welfare concerns (Singh *et al.*, 2021). Early weaning can induce stress in calves, disrupting their natural bonding process and impacting their emotional and physical development (Fernandez-Novo *et al.*, 2020). Furthermore, the absence of maternal milk may compromise their immune system, making them more susceptible to diseases and affecting their overall health and growth (Osorio. 2020).

The welfare implications of early weaning extend beyond immediate health concerns. Calves weaned too early may experience difficulties with digestion and nutrient absorption due to the abrupt transition from milk to solid feed (Jensen *et al.*, 2020). This can lead to developmental delays and increased susceptibility to infections. The stress associated with early weaning has also been linked to behavioral issues and reduced adaptability to farm environments (Blavi *et al.*, 2021). Addressing these welfare concerns requires a re-evaluation of current practices and consideration of alternative approaches that prioritize the health and well-being of dairy calves (Tomlinson *et al.*, 2021). Implementing strategies that allow for a more gradual weaning process could improve calf health and welfare, ultimately benefiting the overall productivity and sustainability of dairy operations (Orihuela *et al.*, 2020).

The safety of dairy products is a significant concern in the dairy industry, particularly regarding contamination by toxic metabolites produced by pathogenic organisms (El-Sayed *et al.*, 2022). These toxins can be generated by bacteria, fungi or other microorganisms that may proliferate in milk and dairy products under certain conditions. The presence of such toxic metabolites poses serious health risks to consumers, potentially leading to food poisoning or other adverse health effects (Gallo *et al.*, 2020). Contaminated dairy products can cause gastrointestinal disturbances, allergic reactions, and in severe cases, more serious health conditions (Grace *et al.*, 2020). Therefore, it is essential for dairy producers to implement rigorous quality control measures to ensure that their products are free from harmful contaminants (Ntuli *et al.*, 2023).

Contamination of dairy products with toxic metabolites often results from inadequate hygiene practices during milk collection, processing, or storage (Akinyemi *et al.*, 2021). Factors such as poor sanitation, improper handling, and insufficient temperature control can contribute to the growth of pathogenic organisms and the production of toxic metabolites (Kamboj *et al.*, 2020). To mitigate these risks, dairy farms must adhere to

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strict sanitation protocols, maintain proper storage conditions, and conduct regular testing for contaminants. Effective monitoring and intervention strategies are crucial in preventing the presence of toxic metabolites in dairy products and ensuring consumer safety (Ntuli *et al.*, 2023).

Pathogenic bacteria are a significant concern in dairy production due to their potential to contaminate milk and dairy products (El-Sayed *et al.*, 2023). Common pathogens associated with milkborne infections include *Mycobacterium bovis*, *Salmonella spp.*, *Corynebacterium spp.*, *Coxiella burnetii*, *Clostridium perfringens*, *Brucella*, *Staphylococcus spp.*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Mycobacterium avium*, *Escherichia coli*, *Listeria spp.*, and various coliforms (Grace *et al.*, 2020; Kumar & Patyal. 2024). These bacteria can cause a range of diseases in both dairy animals and humans, making their presence in dairy products a serious public health issue (Abebe *et al.*, 2020). For example, *Escherichia coli* can cause mastitis in dairy cattle, leading to symptoms that vary from mild irritation to severe infections (Cheng & Han. 2020). The presence of pathogenic bacteria in milk is often linked to poor sanitation and hygiene practices during milk collection, processing, and storage (Nyokabi *et al.*, 2021). Bacteria can be introduced into milk through contaminated equipment, inadequate handling, or environmental sources (Ntuli *et al.*, 2023). Effective control measures, including proper sanitation, regular monitoring, and adherence to hygiene protocols, are essential to prevent bacterial contamination (Kumar *et al.*, 2024). Additionally, implementing measures such as pasteurization can significantly reduce the risk of pathogenic bacteria in dairy products by killing harmful microorganisms (Silva. 2023).

Foodborne infections are a major concern in dairy production, often resulting from the ingestion of food contaminated with viable pathogenic bacteria (Gourama. 2020). Fecal contamination is a significant indicator of the presence of harmful bacteria in dairy products (Souad *et al.*, 2021). Total coliforms, *E. coli* and other enteric bacteria are commonly used as indicators of fecal contamination, reflecting unsanitary conditions during milk handling and processing (El-Prince *et al.*, 2023). The presence of these bacteria in dairy products can lead to serious health issues, including gastrointestinal illnesses and foodborne outbreaks (Grace *et al.*, 2020).

The risk of fecal contamination is particularly high in environments where sanitation practices are inadequate (Chimutu *et al.*, 2024). Contaminated equipment, improper handling of milk and insufficient hygiene can contribute to the presence of fecal bacteria in dairy products. To mitigate these risks, dairy farms must implement strict sanitation protocols, including regular cleaning and disinfection of equipment and facilities (Fusco *et al.*, 2020). Monitoring and testing for indicators of fecal contamination are also essential to ensure the safety of dairy products (Martin *et al.*, 2023).

Public health agencies emphasize the importance of addressing fecal contamination to prevent foodborne illnesses (Todd. 2020). Regular inspection and enforcement of food safety standards are crucial for ensuring that dairy products are free from harmful bacteria (Gandhi *et al.*, 2020). Educating dairy farmers and workers on best practices for hygiene and handling can further reduce the risk of contamination (Alaru *et al.*,

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2022). By prioritizing food safety and adhering to stringent standards, the dairy industry can protect consumer health and prevent foodborne infections (Kumar *et al.*, 2024).

METHODOLOGY

Study Design

The current study was a cross-sectional study.

Study Area

This study was conducted in the Microbiology Laboratory of Institute of Allied Health Sciences, Sarhad University of Science and Information Technology, Peshawar.

Sample Collection

A total of 25 samples were collected from dairy farm located in Agriculture University, Peshawar. These samples were categorized as follows.

Milk Samples

A total of 100 mL of milk were collected from 5 cows and 5 buffaloes, including both clinical and subclinical mastitis cases. Clinical cases involved visibly infected udders, while subclinical cases involved animals with high somatic cell counts but no visible symptoms. Samples were collected in sterile containers and stored at 4°C until processing within 24 hours.

Fecal Samples

Fresh fecal samples were collected from 5 cows and 5 buffaloes directly into sterile containers. Samples were transported in a cool environment and processed within 24 hours to ensure accuracy.

Water samples

A total of 500 mL of water were collected from 5 different drinking sources on the farm using sterile containers. The samples were kept cool and analyzed promptly to assess contamination levels. Table 1. summarizes the collection details for milk, fecal and water samples.

Table. 1. Collection details for milk, fecal and water samples

S. No.	Sample type	Quantity collected	Number of samples
1.	Milk	100 mL per sample	10
2.	Fecal	10 gram per sample	10
3.	Water	500 mL per sample	5

Sample Processing

The collected samples were processed by culturing them on selective media to isolate bacterial species. The samples were plated onto Shigella agar, MacConkey agar and Mannitol Salt Agar (MSA) using sterile techniques to prevent contamination. The inoculated plates were incubated at 37 °C for 24 hours to allow bacterial growth. After

the incubation period, the plates were examined for bacterial colonies (Arbab *et al.*, 2021).

Bacterial Identification

The isolated bacterial pathogens were identified on the basis of morphology, gram staining and several biochemical tests according to standard microbiological techniques (Kabanangi *et al.*, 2021).

Morphological Identification

The isolated colonies were sub cultured on Shigella agar, MacConkey agar and Mannitol Salt Agar (MSA) and incubated at 37 °C for 24 hours. After incubation, the plates were examined for morphological characteristics (Kabanangi *et al.*, 2021).

Gram Staining

The isolated bacteria was differentiated on the basis of Gram staining. It is a method for determining if bacteria are Gram positive or Gram negative. It is a method of identification introduced by Christian Gram for distinguishing Gram negative and Gram-positive isolates.

Reagents for Gram Staining

Gram staining reagents include:

1. Crystal Violet, which is the primary stain.
2. Iodine, which is mordant.
3. Acetone or ethanol, which is decolorizer.
4. Safranin, counter stain

Gram Staining Procedure

Isolated colonies from 24 hrs fresh culture was picked with loop, added to a clean sterile slide A drop of distilled water was added and a smear was formed, which was then dried and heat fixed. Few drops of crystal violet was added to the smear made on slide and after 1 minute the slide was washed with tap water. In the second step, iodine was added to the smear and left for 60 seconds and then washed with water. In the next step, Gram decolorizer was added to the smear for 30 seconds before being rinsed with water. In the final step, safranin was added to the smear for 45 seconds. The slide was dried and observed under microscope (Bunter *et al.*, 2017).

Biochemical Tests

Several biochemical tests including indole, citrate, triple sugar iron (TSI), catalase, urease, coagulase and DNase test was performed to identify the bacterial species isolated.

Indole Test

The isolated bacteria were first inoculated in peptone water and then incubated for 24 hours at 37 °C. The xylene was added to the peptone water and mixed thoroughly to remove indole. After this, Kovac reagent was added to the tubes. The formation of a pink colored ring on the surface of the tube showed positive result (Mohammed *et al.*,

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Citrate Test

The isolated bacterial colonies were gently inoculated on simmon citrate agar slants using a sterilized wire loop and incubated at 37°C for 24 hours. After incubation, the slants were observed for a color change from green to blue which showed positive results (Holt *et al.*, 2018).

Triple Sugar Iron (TSI) Test

Triple Sugar Iron (TSI) slants were prepared. A sterilized wire loop was used to pick and streak a single colony across the surface of the slant. The tubes were incubated for 24 hours at 37°C and were examined for a positive reaction (Jathar *et al.*, 2021).

Urease Test

The aim of Urease test is to identify an organism's ability to breakdown urea into carbon dioxide and ammonia using urease enzyme. For the Urease test, inoculate a urea-agar slant with an overnight fresh culture. The tubes were incubated for 48 hours to 7 days at 37 °C with the cap loosely on. After that, observe the tubes for the appearance of pink color. Urease positive organisms develop a strong pink color between 15 minutes to 24 hours, while urease negative organisms do not change color (Aldaw, 2022).

Catalase Test

Catalase test identifies the synthesis of catalase enzyme in aerobic organisms. Catalase is an enzyme that converts hydrogen peroxide into water and oxygen. Few drops of hydrogen peroxide was taken and transferred to the slide. An isolated bacterial colony was picked with loop and transferred to the slide. Bubbles were formed, which indicted positive result (Holt *et al.*, 2018).

DNase Test

DNase media was prepared and isolated bacterial colonies were cultured on the test area and incubated for 24 hours at 37°C. After 24 hours of incubation, the plates were rinsed with 1 N HCl. Clear zones surrounding the bacterial colonies indicate positive result (Kateete *et al.*, 2019).

Coagulase Test

An isolated bacterial colony was picked with loop and transferred to the slide. After that, human plasma was added to the smear, which was mixed well. The slide was observed for clot formation. Clot formation indicated positive result (Holt *et al.*, 2018).

Antibiotic Susceptibility Testing

The confirmed bacterial isolates were tested for antibiotic susceptibility by Kirby-Bauer disc diffusion method against frequently used antibiotics belonging to various groups (CLSI, 2022). Inoculum for susceptibility testing was prepared by suspending three to five well isolated colonies from nutrient agar plate into an eppendorf tube containing normal saline. The turbidity of the inoculum was adjusted by comparing it with 0.5 McFarland standard. Bacterial lawn was prepared by dipping a sterile cotton swab into

the eppendorf tube. Excess fluid was squeezed against the wall of the eppendorf tube. The swab was streaked onto the Muller Hinton agar plate and left for five minutes to dry. The antibiotics were then aseptically placed on MHA plate and incubated for 24 hours at 37 °C. After incubation, the zones of inhibition were measured with the help of scale (Arbab *et al.*, 2021).

The 12 different antimicrobial agents including ceftriaxone (30 µg), piperacillin/tazobactam (110 µg), ciprofloxacin (5 µg), azithromycin (15 µg), doxycycline (30 µg), sulphamethazole/trimethoprim (23.75 µg), amikacin (30 µg), gentamicin (10 µg), methicillin (5 µg), oxacillin (1 µg), vancomycin (30 µg), linezolid (30 µg) and clindamycin (2 µg) were used and results were recorded following the guidelines of CLSI.

RESULTS

Total Samples Processed

A total of 25 samples including 10 milk samples, 10 fecal samples and 5 water samples were processed in the the Microbiology Laboratory of Institute of Allied Health Sciences, Sarhad University of Science and Information Technology, Peshawar. For the milk samples, a total of 10 samples were tested and *Staphylococci* and *Escherichia species* were identified as the suspected bacteria. In the case of the fecal samples, also comprising 10 samples, both *Salmonella* and *Escherichia coli* were detected. Furthermore, among the 5 water samples, *Escherichia coli* was the suspected bacterium.

Table 1. Summary of sample analysis

S. No.	Sample Type	No. of samples	Suspected bacteria
1.	Milk	10	<i>Staphylococci</i> and <i>E. coli</i>
2.	Fecal	10	<i>Salmonella</i> and <i>E. coli</i>
3.	Water	5	<i>E. coli</i>

Initial Bacterial Characterization

Out of 25 samples, 6 (24 %) of the bacterial isolates were gram positive and 19 (76 %) of the isolates were gram negative. Among them, *E. coli* was the most predominant isolate (48 %), followed by *Salmonella* spp. (28 %) and *Staphylococcus* spp (24 %). The suspected isolated gram positive and gram negative bacteria are shown in Table 2.

Table 2. Initial Bacterial Characterization

S. No.	Suspected Bacteria	No. of Isolates	Isolation Rate
1.	<i>Staphylococcus</i> spp.	6	24 %
2.	<i>Salmonella</i> spp.	7	28 %
3.	<i>Escherichia coli</i>	12	48 %

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Total	25	100 %
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Identification of the suspected bacteria

Morphological identification

Salmonella species formed colorless or pale yellow colonies on shigella agar. *Staphylococcus* species formed bright yellow colored colonies on MSA media and *E. coli* formed pink, flat, mucoid colonies on macConkey agar media.

Gram Staining

The suspected *Salmonella*, *Staphylococcus* and *E. coli* species were further identified through gram staining. The suspected bacteria with their colony morphology are shown in Table 3.

Table 3. Gram positive and gram-negative bacterial isolates with their colony morphology

S. No.	Suspected bacterial species	Culture media	Morphological appearance	Gram reaction
1.	<i>Staphylococcus</i>	Mannitol salt agar	Colorless or pale yellow colonies	Gram-positiv
2.	<i>Salmonella</i>	Shigella agar	Colorless or pale yellow colonies	Gram-negative
3.	<i>E. coli</i>	MacConkey agar	Pink, flat, mucoid colonies	Gram-negative

Biochemical Tests

Several biochemical tests performed to identify the isolated gram-positive and gram-negative bacteria with their results are shown in Table 4. Different bacterial species were identified based on various biochemical tests.

Table 4. Biochemical tests with their results

Biochemical Tests							
Predicted Bacteria	Indole	Citrate	TSI	Urease	Catalase	DNase	Coagulase
<i>Staphylococcus</i> spp.	- ve	- ve	- ve	- ve	- ve	+ ve	+ ve
<i>Salmonella</i> spp.	-ve	+ ve	Acid slant/alkaline butt	- ve	+ ve	- ve	- ve

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<i>E. coli</i>	+ ve	-ve	+ ve	-ve	-ve	-ve	-ve
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Antibiotic Susceptibility Testing

Antibiotic Susceptibility of Gram-Positive Bacteria

The antibiotic susceptibility testing was performed on a total of 6 (24 %) *Staphylococci* isolates. Among them, 5 (83.33 %) of isolates were methicillin resistant *S. aureus* (MRSA). Vancomycin and Linezolid was the most effective antibiotic against all *S. aureus* isolates. The susceptibility results are listed in Table 5.

Table 5. Antibigram of gram-positive bacterial isolates

S. No.	Antibacterial Drug	Zone of Inhibition N=6		
		Resistant n (%)	Intermediate n (%)	Sensitive n (%)
1.	Methicillin	5 (83.33 %)	-----	1 (16.66 %)
2.	Oxacillin	5 (83.33 %)	-----	1 (16.66 %)
3.	Clindamycin	3 (50 %)	1 (16.66 %)	2 (33.33 %)
4.	Vancomycin	-----	-----	6 (100 %)
5.	Linezolid	1 (16.66 %)	-----	5 (83.33 %)

Antibiotic Susceptibility of Gram-Negative Bacteria

The antibiotic susceptibility testing was performed for a total of 19 (76 %) gram negative bacterial isolates. All the gram negative isolates were MDR, showing resistance to multiple drugs. Piperacillin/Tazobactam was the most sensitive antibiotic against the isolated gram negative bacteria. The results are listed in Table 6.

Table 6. Antibiotic susceptibility testing of gram-negative bacterial isolates

S. No.	Antibacterial Drug	Zone of Inhibition N=19		
		Resistant n (%)	Intermediate n (%)	Sensitive n (%)
1.	Ceftriaxone	2 (10.52 %)	-----	17 (89.47 %)

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2.	Piperacillin-Tazobactam	1 (5.26 %)	-----	18 (72 %)
3.	Ciprofloxacin	3 (15.78 %)	2 (10.52 %)	14 (73.68 %)
4.	Azithromycin	14 (73.68 %)	1 (5.26 %)	4 (21.05 %)
5.	Doxycycline	-----	2 (10.52 %)	17 (89.47 %)
6.	Trimethoprim-Sulfamethoxazole	-----	4 (21.05 %)	15 (78.94 %)
7.	Gentamicin	11 (57.89 %)	-----	8 (42.10 %)
8.	Amikacin	7 (36.84 %)		12 (63.15 %)

DISCUSSION

Pathogenic bacteria in dairy farm animals can lead to serious health issues and affect the quality of milk produced. These bacteria can cause infections in animals, leading to diseases that not only compromise animal welfare but also pose risks to human health through contaminated milk (Owusu-Kwarteng *et al.*, 2020). Identifying and managing these pathogens is crucial for ensuring the overall health of dairy herds and maintaining high standards of food safety (Ledo *et al.*, 2020). Proper hygiene practices, effective treatment and monitoring are essential to control the spread of these harmful bacteria and protect both animals and consumers (Scavizzi *et al.*, 2022). Therefore, this study aimed to assess the prevalence of pathogenic bacteria from dairy farm.

In the current study, a total of 25 samples including 10 milk, 10 fecal, and 5 water samples were processed. In the milk samples, *Staphylococci* and *Escherichia coli* were identified. The fecal samples revealed the presence of *Salmonella* and *Escherichia coli*. Whereas, *Escherichia coli* was detected in water samples. Similarly, Geletu *et al.* (2022) isolated *S. aureus*, *E. coli*, and *Salmonella* from both fresh cow milk and fecal samples. Gezahegn *et al.* (2023) also identified *S. aureus*, *E. coli* and *Salmonella* in their study. Dadi *et al.* (2020) reported the presence of *E. coli* and *Salmonella* in milk samples from a dairy farm in Ethiopia. Additionally, Hunduma *et al.* (2023) found *Salmonella* in composite milk samples from dairy cows.

In the current study, of the 25 samples analyzed, 6 (24%) of the bacterial isolates were Gram-positive, while 19 (76%) were Gram-negative. This distribution mirrors findings reported by Otenga (2023), who similarly identified both Gram-positive and Gram-negative bacterial isolates in their research. Allied. (2021) also reported the presence of

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both types of bacteria in their study. Additionally, Yimana and Bekele (2022) focused on Gram-positive bacteria specifically isolated from bovine milk samples, highlighting the variability in bacterial profiles depending on the sample type and source. These observations Highlight the diverse nature of bacterial populations encountered in dairy farm environments and the importance of comprehensive microbial analysis.

In the current study, *E. coli* was identified as the most predominant isolate, accounting for 48% of the samples, followed by *Salmonella spp.* (28 %) and *Staphylococcus spp.* (24 %). This finding is consistent with Hunduma *et al.* (2023), who reported *E. coli* (33.4 %) in cow milk samples. Similarly, Bag *et al.* (2021) isolated *E. coli* from 35.8 % of mastitis milk samples and Madani *et al.* (2022) identified pathogenic strains in 30 % of traditional dairy products.

In the current study, antibiotic susceptibility, testing was conducted on 6 (24%) *S. aureus* isolates. Among these, 5 (83.33 %) were identified as Methicillin-resistant *S. aureus* (MRSA). Vancomycin and Linezolid were found to be the most effective antibiotics against all *S. aureus* isolates. This aligns with Girmay *et al.* (2020), who reported 33.33 % MRSA with susceptibility to vancomycin. Additionally, Mphahlele *et al.* (2020) and Zhao *et al.* (2021) both found Linezolid and Vancomycin to be the most effective antibiotics for MRSA.

All Gram-negative isolates (76 %) in this study were multidrug-resistant (MDR), demonstrating resistance to multiple antibiotics. Piperacillin/Tazobactam emerged as the most effective antibiotic against these Gram-negative bacteria. This is consistent with Bag *et al.* (2021), which found 84.2 % of isolates to be multidrug-resistant and Tahar *et al.* (2020), who reported multidrug resistance in 38.4 % of isolates. These findings highlight the urgent need for continuous monitoring of antibiotic resistance patterns and the development of targeted treatment strategies to manage multidrug-resistant infections effectively.

Conclusion

In conclusion, the current study successfully identified and characterized bacterial pathogens from milk, fecal and water samples, revealing *E. coli* as the most prevalent isolate, followed by *Salmonella* and *Staphylococcus spp.* Antibiotic susceptibility testing showed a high prevalence of multidrug resistance among Gram-negative bacteria, with Piperacillin/Tazobactam being the most effective treatment. In contrast, Vancomycin and Linezolid were highly effective against *Staphylococci* isolates.

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