





# Assessment of antibiotic resistant profile of coliform and *Staphylococcus* spp. isolated from milk from Kathmandu valley

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## Abstract

Antimicrobial resistance (AMR) among milk pathogens is increasing, which is a serious threat to consumers' health. Therefore, this study aims to assess the current antibiotic profile of coliforms and *Staphylococcus* spp. in milk samples. For this, thirty milk samples were collected from various locations in Kathmandu district. Isolation and enumeration were done on selective media using streak-plate and pour-plate techniques, respectively. Antibiotic susceptibility testing (AST) was done by the Kirby-Bauer disk diffusion method. A total of 48 bacteria were isolated, of which 31 were coliform and 17 *Staphylococcus* spp. Among the coliforms, *Klebsiella* spp. (n=17, 54.84%) was the most predominant in both raw (n=12, 70.6%) and pasteurized milk (n=5, 29.4%), followed by *E. coli* and *Citrobacter* spp. While for *Staphylococcus* spp., 15 (88.24%) were *S. aureus* and 2 (11.76%) were coagulase negative *Staphylococcus* (CONS). *S. aureus* was dominant in raw milk (n=13) rather than pasteurized milk (n=2). The AST of coliforms showed higher resistance towards ampicillin (96.75%), followed by cefoxitin, ciprofloxacin, nalidixic acid, nitrofurantoin, piperacillin, co-trimoxazole, ceftriaxone, chloramphenicol, and amikacin in descending order. In the case of *S. aureus*, higher resistance was observed for penicillin G (100.00%), followed by cefoxitin, ampicillin, ceftriaxone, tetracycline, ciprofloxacin, and amikacin. Further, 12 (70.53%) *S. aureus* were confirmed as methicillin-resistant *S. aureus* (MRSA). And a total of 10 (32.25%) coliforms and 9 (52.95%) *S. aureus* were identified as multiple drug resistant (MDR) strains. Thus, it can be concluded that antibiotic resistance among milk isolates of the coliform and *Staphylococcus* spp. is highly prevalent, and these can be a potential source of incurable milk-borne infections. Thus, routine assessment of microbial quality as well as AMR surveillance should be done on milk isolates to ensure the safety of consumer's health.

**Keywords:** Multidrug resistant, Methicillin Resistant *S. aureus* (MRSA), Antibiotic susceptibility testing, *E. coli*, *S. aureus*

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## Introduction

Milk is considered a complete meal; however, it is likely to cause health hazards to customers if consumed with microbial contaminants [1]. A number of milk-borne epidemics and outbreaks, such as diphtheria, dysentery, typhoid, etc., have occurred through the consumption of milk and its products by humans [2]. The presence of food pathogens like *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* in milk and milk products is responsible for food-borne infections ranging from mild to serious cases, even leading to death [3]. Additionally, AMR among these food pathogens is on the rise [3-5] and has resulted in an increased number of hospitalizations, which in turn has increased morbidity and mortality [6]. According to the WHO, at least 700,000 people die each year due to drug-resistant diseases, which could escalate to 10 million deaths per year by 2050 [7]. Such an escalation of AMR is a serious concern, specifically the resistance towards the available drugs [3-5, 8], which may limit the treatment options.

Several studies around the world have reported the presence of AMR coliforms such as *E. coli* in milk samples

[1, 4,5, 8- 10], and the results of those studies have clearly shown the rising trend of AMR among milk pathogens [4-5, 8, 10]. In particular, the most common antibiotic resistance conferred by these coliforms is toward the  $\beta$ -lactam class of antibiotic [4-5, 8]. Similarly, in the case of *Staphylococcus*, AMR strains [3-5, 8-10] as well as toxin-producing strains [11, 12] have been widely reported in milk samples. The random practice of using antimicrobials to promote livestock growth or for the treatment of disease may be a contributing factor in the emergence of AMR. Such uncontrolled antibiotic usage can lead to resistance in microorganisms, which makes antimicrobials less effective as medicine for humans and livestock [7].

In Nepal, the most common milk isolates showing higher AMR are notably *E. coli* [1, 8, 13] and *Staphylococcus* spp. [1, 13-14]. But due to the lack of focus on milk pathogens, studies related to AMR milk pathogens are limited in Nepal [13]. But evidently, AMR bacteria are unceasingly evolving and disseminating through the overuse and misuse of antibiotics both in humans and veterinary animals [1, 8, 13-14]. And notably, such AMR bacteria

pose a high risk to human health and are becoming an enormous public health challenge throughout the world, including Nepal. Therefore, it is essential to know the current trend of antimicrobial resistance among the pathogens that are prevalent in food to ensure the safety of consumers. This study was aimed at assessing the current trend of antibiotic resistance in coliform and *Staphylococcus* spp. that prevails in pasteurized and raw milks of Kathmandu Valley.

## Materials and methods

### Sampling and transportation

A cross-sectional study was conducted between January 2021 and June 2021. A total of 30 milk samples (15 raw and 15 pasteurized) were tested, which were collected from 5 different locations in Kathmandu district (Kalanki, Balaju, Swayambhu, Thamel, and Budhanilkantha). Pasteurized milk was collected as per the manufacturer's sale package (a 500-ml pack) available from retail shops, while raw milk samples were collected in a sterile screw-capped bottle from farms. Both samples were transported to the laboratory within 2 hours, maintaining the cold chain. Sample collection as well as transportation were done in compliance with the guidelines stated in the bacteriological analytical manual [15].

### Isolation and enumeration

Each sample was first serially diluted up to  $10^{-8}$ , and then the diluents were cultured on specific media plates using pour-plate techniques. Plate count agar (PCA) plates, violet red bile agar (VRBA) plates, and mannitol salt agar (MSA) plates were used for determining the total bacterial count (TBC), total coliform count (TCC), and total staphylococcal count (TSC), respectively [16]. For thermotolerant *E. coli*, VRBA plates were incubated at 44 °C and enumerated for fecal coliform count (FCC). All the enumeration results were interpreted following BIS (Bureau of Indian Standards) [17] and DFTQC (Department of Food Technology and Quality Control) [18] quality guidelines. For isolation, distinct colonies of coliform from VRBA plates and distinct colonies of

*Staphylococcus* spp. from MSA plates were subcultured on nutrient agar, respectively, and identified based on their biochemical characteristics using IMViC test for coliform and coagulase test for *Staphylococcus aureus* [19].

### Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed on all the isolates following the Kirby-Bauer disc diffusion method. Twelve different antibiotics, namely ciprofloxacin (CIP 5 mcg), ceftriaxone (CTR 30 mcg), ampicillin (AMP 10 mcg), cefoxitin (CX 30 mcg), nalidixic acid (NA 30 mcg), nitrofurantoin (NIT 300 mcg), tetracycline (TE 30 mcg), piperacillin/tazobactam (PTZ 100 mcg), cotrimoxazole (COT 25 mcg), levofloxacin (LE 5mcg), chloramphenicol (C 30mcg), and amikacin (AK 30mcg) were used for coliforms. however, 10 different antibiotics, namely ciprofloxacin (CIP 5 mcg), ceftriaxone (CTR 30 mcg), ampicillin (AMP 10 mcg), cefoxitin (CX 30 mcg), nalidixic acid (NA 30 mcg), tetracycline (TE 30 mcg), piperacillin/tazobactam (PTZ 100 mcg), co-trimoxazole (COT 25 mcg), levofloxacin (LE 5 mcg), chloramphenicol (C 30mcg), amikacin (AK 30mcg) and penicillin G (P 10 mcg) were used for *Staphylococcus* spp. The choice of antibiotics was based on treatment regime while other class of antibiotics beside treatment regime were also included, to observe the correlation among different classes.

All the results of the zone of inhibition were interpreted as per the recommendation guidelines of CLSI [20]. Also, all the intermediate isolates were placed under the resistant category during the statistical analysis. Isolates with resistance to three or more different classes of antibiotics were considered MDR. While *S. aureus* isolates showing resistance to cefoxitin were considered MRSA. Additionally, cultures of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used for the quality assurance of the disk diffusion method and AST result interpretation.

### Data collection and analysis

The data was entered in MS Excel. The frequency distribution, normal distribution testing, ]variance analysis, and correlation were done using SPSS (version

**Table 1.** Descriptive Statistics of Bacterial Count based on sample type (N=30)

	Raw milk (N= 15)			Pasteurized milk (N = 15)		
	Minimum $\times 10^5$ CFU/ml	Maximum $\times 10^5$ CFU/ml	Mean $\pm$ S.D $\times 10^5$ CFU/ml	Minimum $\times 10^5$ CFU/ml	Maximum $\times 10^5$ CFU/ml	Mean $\pm$ S.D $\times 10^5$ CFU/ml
TBC	0.033	1.798	0.443 $\pm$ 0.398	0.017	0.4	0.110 $\pm$ 0.101
TCC	0	0.095	0.028 $\pm$ 0.027	0	0.07	0.013 $\pm$ 0.023
FCC	0	0.081	0.019 $\pm$ 0.026	0	0.085	0.012 $\pm$ 0.028
TSC	0	0.45	0.114 $\pm$ 0.123	0	0.03	0.007 $\pm$ 0.012

Note: TBC=total bacterial count, TCC=total coliform count, FCC=fecal coliform count. and TSC=total staphylococcal count.

20). The significance was measured at 95% confidence intervals. The resistance profile was analyzed using WHONET 2020, and the results were interpreted accordingly.

### Result

The TBC of 15 raw milk samples ranged from  $3.25 \times 10^7$  to  $1.798 \times 10^5$  CFU/ml. Since all the raw samples had TBC lower than the recommended BIS guideline [17], all of them were graded as good quality raw milk. Likewise, the TBC of 15 pasteurized milk samples ranged from  $0.0173 \times 10^5$  to  $0.4 \times 10^5$  CFU/ml. But only 14(93%) samples were within the acceptable range of  $3 \times 10^4$  CFU/ml based on the BIS guideline for pasteurized milk [17].

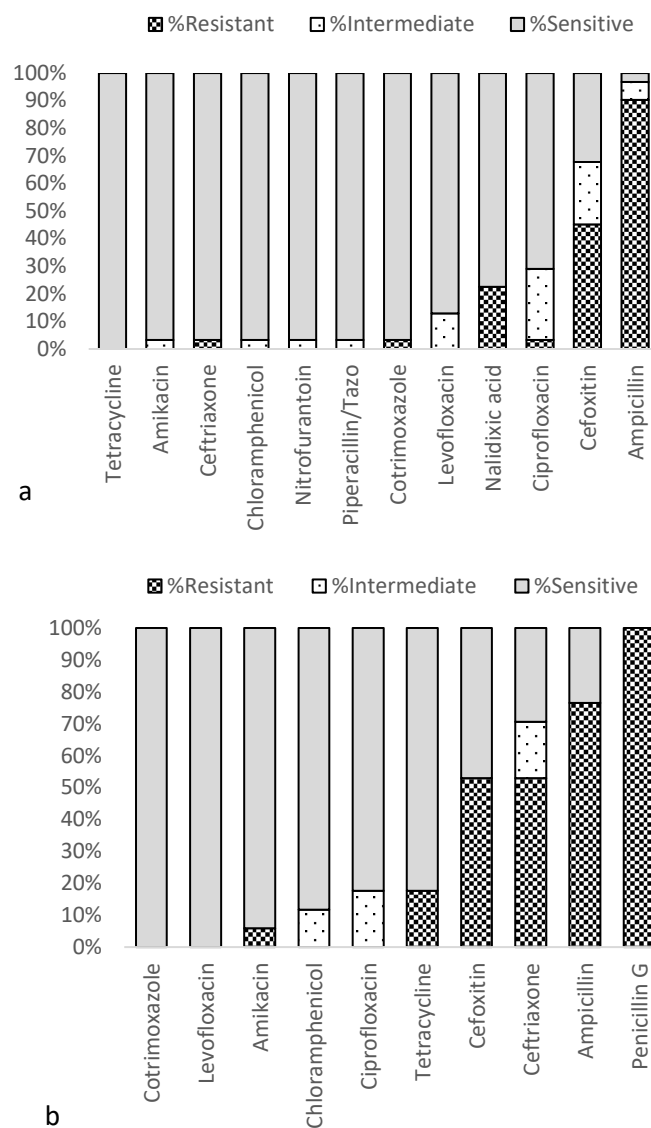
Further, the TCC of all milk samples ranged from  $0.0037 \times 10^5$  to  $0.56 \times 10^5$  CFU/ml. So, 3(20%) raw samples and 9(60%) pasteurized samples were within TCC acceptable limits [17, 18]. Similarly, the FCC of all milk ranged from  $0.0036 \times 10^5$  to  $0.19 \times 10^5$  CFU/ml. And according to the guideline [17, 18], 6(40%) raw sample and 11(73.33%) pasteurized sample were within the FCC acceptable limit (Coliform should be zero). The TSC of 15 raw milk samples ranged from  $3.0 \times 10^3$  CFU/ml to  $45 \times 10^3$  CFU/ml. The TSC of 15 pasteurized milk samples ranged from  $2.07 \times 10^3$  CFU/ml to  $3.0 \times 10^3$  CFU/ml. Sample wise descriptive statistics of bacterial count is provided in **Table 1**.

### Identification of milk isolates

A total of 48 bacterial isolates were isolated, among them 31(64.53%) were coliforms (of which 13 were thermotolerant coliforms) while 17(35.47%) were *Staphylococcus* spp. Further, of those 31 isolated coliforms, 17(54.84%) were identified as *Klebsiella* spp., 13(41.94%) were *Escherichia coli* and 1(3.22%) was *Citrobacter* spp (**Table 2**). Likewise, for *Staphylococcus* spp., out of 17 isolates 15(88.24%) were identified as *S. aureus* and 2(11.76%) were CONS (**Table 2**).

### Antibiotic susceptibility profile

All 48 isolates were subjected to AST. The distribution of antibiotic susceptibility of coliforms and *Staphylococcus* spp. is given in **Figure 1**.



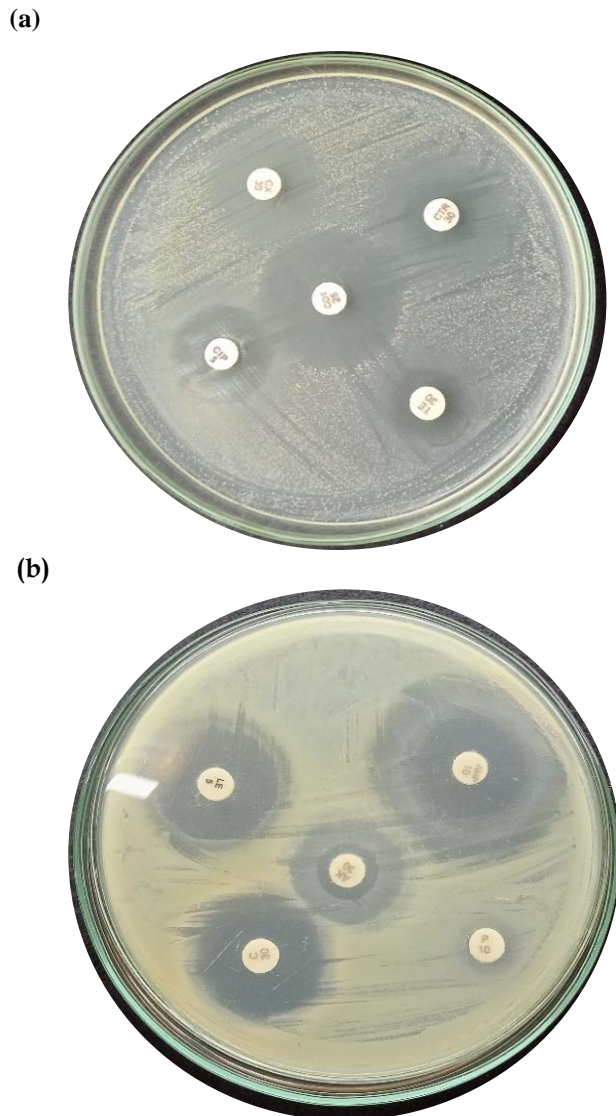
**Figure 1.** The antibiotic susceptibility pattern of (a) Coliforms and (b) *Staphylococcus* spp.

In case of *E. coli* (n=13), 100% susceptibility was observed toward chloramphenicol, tetracycline, cotrimoxazole, piperacillin, nitrofurantoin, ceftriaxone. while the susceptibility towards amikacin, levofloxacin, ciprofloxacin, nalidixic acid, cefoxitin and ampicillin were 92.31%, 76.99%, 69.22%, 69.22%, 46.12% and 7.67% respectively (**Figure 2**).

**Table 2:** Sample wise distribution of isolates (n= 48)

		Raw (N= 15)		Pasteurized (N= 15)		Total
		Count	%	Count	%	
<b>Gram-negative (n=31)</b>	<i>E. coli</i>	9	69.2%	4	30.8%	13
	<i>Klebsiella</i> spp.	12	70.6%	5	29.4%	17
	<i>Citrobacter</i> spp.	0	0	1	100%	1
<b>Gram-positive (n=17)</b>	<i>S. aureus</i>	13	86.7%	2	13.3%	15
	CONS	0	0	2	100.0%	2
<b>Total isolates</b>		34	70.8%	14	29.2%	48





**Figure 2:** AST plate of (a) *E. coli* showing ZOI to CIP (Ciprofloxacin), CTR (Ceftriaxone), TE (Tetracycline), CX (Cefoxitin), and COT (Cotrimoxazole) (b) *S. aureus* showing ZOI to C (Chloramphenicol), P (Penicillin G), LE (Levofloxacin), AK (Amikacin) and AMP (Ampicillin)

Likewise, for *Klebsiella* spp. (n=17), 100% susceptibility was observed toward amikacin and tetracycline. while the susceptibility towards levofloxacin, cotrimoxazole, nitrofurantoin, piperacillin, chloramphenicol and ceftriaxone was 94.16%. Further, susceptibility toward nalidixic acid, ciprofloxacin and cefoxitin were 82.32%, 76.42% and 23.53% respectively. While 100% resistant was observed for ampicillin.

Similarly, in case of *Citrobacter* spp. (n=1), 100% susceptibility was observed toward levofloxacin, nalidixic acid, amikacin, ceftriaxone, chloramphenicol, nitrofurantoin, piperacillin, tetracycline and cotrimoxazole while, 100% resistant was observed for ampicillin, ciprofloxacin and cefoxitin.

In case of *S. aureus* (n=15), 100% was observed toward levofloxacin, cotrimoxazole, and chloramphenicol while

none of the isolates showed susceptibility toward penicillin G. Also, the susceptibility for amikacin, ciprofloxacin, tetracycline, ceftriaxone, ampicillin and cefoxitin were 93.33%, 80%, 80%, 46.67%, 26.67% and 20% respectively.

While for CONS (n=2), all the isolates showed 100% susceptibility toward levofloxacin, cotrimoxazole, tetracycline, amikacin and ciprofloxacin while none of the isolates showed susceptibility toward penicillin G, chloramphenicol, ceftriaxone, ampicillin and cefoxitin.

A total of 19 MDR were detected in this study, of which 10 (35.25%) were coliforms and 9 (52.95%) were *Staphylococcus* spp. Among 10 MDR coliform, 5 (16.13%) were thermotolerant coliform. Additionally, 12 (70.53%) isolates of *S. aureus* were confirmed as MRSA based on their resistance toward cefoxitin (S<sub>I</sub>≥25mm). Resistance profile of isolates based on priority is given below **Table 3**.

## Discussion

Milk is a staple of an average person's diet, but good-quality milk is readily available in the market, as a noteworthy prevalence of milk pathogens has been reported in Nepal [1, 8, 21-23]. In this study, TBC of all the raw milk samples and 93.33% of pasteurized milk samples were within the acceptable limit of the recommended guidelines [17]. This result complies with the study done by DFTQC showing that more than 50% of the milk is microbiologically safe for consumption. But this assumption is solely based on results from TBC, and results from TBC alone may not be a reliable way to determine the quality of milk, so additional microbial parameter testing should be done. Additionally, in this study, TCC percentages were higher in raw samples and lower in pasteurized samples, which may be due to the entry of coliform via water. Even the fecal coliforms (thermotolerant) were present in approximately half of the total samples (43.33%), which indicates the presence of sewage contamination of a waterway and the possibility of the presence of another pathogenic organism. Since food guidelines suggest that coliform should not be present in food sample [17, 18], in this study, the presence of coliform and fecal coliform in both raw and pasteurized milk samples (**Table 1**) suggests that the tested milk samples are questionable in quality. Similar high occurrence of coliform in milk was reported by Rai et al. [8] and Phattepuri et al. [10].

Both Gram-positive and Gram-negative bacteria were isolated in this study. The Gram-negative bacteria were in higher proportion compared to the Gram-positive bacteria (**Table 2**). And among the coliforms, *Klebsiella*

**Table 3.** Resistant profile of isolates based on priority

Type of sample	Resistance antibiotics	No	Organism	Priority list
Pasteurized	AK <sup>R</sup> AMP <sup>R</sup> CX <sup>R</sup>	1	<i>E. coli</i>	Medium Priority
Pasteurized	AMP <sup>R</sup> CX <sup>R</sup> CIP <sup>R</sup> CTR <sup>R</sup> CR <sup>R</sup> NA <sup>R</sup>	1	<i>Klebsiella</i> spp.	Medium Priority
Pasteurized	AMP <sup>R</sup> CIP <sup>R</sup> COT <sup>R</sup> LER	1	<i>Klebsiella</i> spp.	Medium Priority
Raw	AMP <sup>R</sup> CX <sup>R</sup> CIP <sup>R</sup> CTR <sup>R</sup>	2	<i>S. aureus</i>	Medium Priority
Raw	AK <sup>R</sup> AMP <sup>R</sup> CX <sup>R</sup> CIP <sup>R</sup> CTR <sup>R</sup>	1	<i>S. aureus</i>	Medium Priority
Raw	AMP <sup>R</sup> CX <sup>R</sup> CTR <sup>R</sup>	4	<i>S. aureus</i>	Medium Priority

spp. was predominant in this study, followed by *E. coli* and *Citrobacter* spp. (Table 2). Both *E. coli* and *Klebsiella* spp. are intestinal commensal and are abundantly found in the environment [24] so their presence in contaminated milk is apparent.

Notably, *E. coli* was present in higher number of sample (41.94%), which is lower than the findings of Rai et al. [8] and Phattepuri et al. [10]. Such a higher prevalence of coliforms may be due to their abundant occurrence in the environmental settings and easy access into milk during collection, transport or storage.

In the case of *Staphylococcus* spp., their count showed a significant positive correlation between raw and pasteurized milk statistically ( $p < 0.05$ ), which justifies the higher presence of TSC in raw milk than pasteurized milk. A similar prevalence of *S. aureus* in raw milk was found in the study of Rai et al. [8], while a contrary result was shown in Limbu et al. [22]. The higher proportion of *Staphylococcus* spp. in raw milk may be due to the fact that they are unprocessed samples, and bacteria like *Staphylococcus*, which are widely present on the human and environmental surface, are likely to get their way into the milk through milk handlers, from utensils, from udder etc. very easily. While a lower fraction of *S. aureus* was observed in pasteurized milk, which is similar to the report from Arjyal et al. [19]. Irrespective of sample type, the occurrence of *S. aureus* in milk has been reported in several studies over the decade [3-5, 8-12]. Since pasteurized milk samples are processed samples and these bacteria can be effectively killed by pasteurization, it is likely that their count decreased due to heat treatment. However, irrespective of heat treatment, the enterotoxins produced by *S. aureus* retain their biological activity, which is a matter of concern and can be hazardous to consumers [11, 12]. Literature suggests that

even a small dose of Staphylococcal enterotoxin (less than 1mg) can cause illness in people, and such a level of toxicity can be seen when the *S. aureus* population exceeds  $10^5$  CFU/g of food [25].

The antibiotic susceptibility test result indicated that *Staphylococcus* spp. showed higher resistance than the coliforms (Figure 1). Specifically, the coliform isolates (90.33%) were resistant to ampicillin, which was in compliance with the findings of Rai et al. [8] and Badri et al. [24]. At strain level, *Klebsiella* spp. (100%) was resistant to ampicillin and similar higher resistance was reported in the study of Badri et al. [24]. Further, *E. coli* were mainly resistant to ampicillin, which matches the findings of Rizal et al. [1], Phattepuri et al. [10] and Badri et al. [24] (Figure 2). Not just in this study but the prevalence of drug-resistant bacteria in food pathogens has sharply grown globally [4] and this rise is due to their high potency in AMR gene acquisition and spread [5].

While in the case of *Staphylococcus* spp., though 100% susceptibility was observed toward levofloxacin, cotrimoxazole, and chloramphenicol, higher resistance was shown toward penicillin G and cefoxitin, which is noteworthy. The MRSA prevalence of this study was higher than the reports of Rizal et al. [1] and Rai et al. [8], while lower than the study report of Parajuli et al. [21]. As cefoxitin resistance is indicative of MRSA, the presence of such a high prevalence of cefoxitin resistance is alarming, as high morbidity and mortality rates from MRSA are still an undeniable clinical threat [6]. Besides cefoxitin, in this study, *S. aureus* was further resistant to penicillin G, which was similar to Phattepuri et al. [10] but contradictory to Rizal et al. [1], where the isolates showed mainly resistance to ampicillin and chloramphenicol. Apart from cefoxitin, this study also detected a higher level of resistance among the lactam classes of antibiotics (penicillin G and ampicillin) in *S. aureus*, which is noteworthy too. Such resistance is attributed to the production of beta-lactamase, an enzyme that inactivates penicillin and related antimicrobials. Around 50% of mastitis-causing *S. aureus* strains produce beta-lactamase [26]. And the literature suggests that this lactam resistance is easily developed by evolution or gene transfer [26]. Further, uncontrolled usage of these antibiotics among livestock creates a stressful environment for bacteria, contributing to the development of AMR.

MDR was detected in both coliforms and *Staphylococcus* spp. In the case of coliforms, the MDR and their antibiogram analysis indicated that out of 10 MDR, 3 were of medium priority (Table 3). Even out of 10 MDR bacteria, 3 were thermotolerant, which suggests that they

are of fecal origin. However, the prevalence of MDR in *E. coli* was less than the finding of Rai et al. [8]. Whereas, in the case of *Staphylococcus* spp., the proportion of MDR in this study resembled the finding of Rai et al. [8]. In this study, it was observed that the MDR isolate was more prevalent in raw milk than in pasteurized milk. The reason for this may be due to the fact that raw milk has a higher level of environmental exposure, which increases the likelihood that resistant isolates may end up in raw milk, and also the fact that raw milk samples were unprocessed.

The growing resistance among milk isolates and the existence of MDR raise significant concerns as they may lead to treatment failure and limit therapy options in humans and animals. So undoubtedly, the presence of AMR bacteria, including the MDR bacteria, MRSA, in milk can pose a serious health risk to consumers [3-5, 13-14, 24]. In order to accurately detect and successfully treat milk-borne diseases, routine monitoring of milk pathogens' resistance profiles should be commenced, along with an evaluation of the milk's microbiological quality, in order to protect consumers.

## Conclusion

In conclusion, potential bacterial pathogens like *E. coli*, *Klebsiella* spp and *S. aureus* were highly prevalent in both raw and pasteurized milk samples. And higher prevalence of antibiotic resistance was observed within them. Such occurrence of antibiotic resistance particularly the multiple antibiotics resistant, within such potent bacterial isolates in milk sample is a concerning situation. Therefore, a trend analysis and control strategy for AMR could be an alternative to intervene the emergence, evolution and dissemination of AMR in dairy products, which will ultimately ensure the safety of the consumer's health.

## Ethical Approval and Consent

A brief detail of this research study was provided to the farm owner and a verbal consent was obtained before sampling. This study was carried out with the approval from the concerned authorities.

## Availability of data

The data can be made available upon request.

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