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Prevalence and Risk Factors of Multidrug Resistant (MDR) *Escherichia coli* Isolated from Milk of Small Scale Dairy Buffaloes in Rupandehi, Nepal

Anil Gautam [1,*], Sirjan Bastola [1], Keshav Lamsal [1], Krishna Kaphle [1], Prajjwal Shrestha [2], Shambhu Shah [1] and Deepak Subedi [3,*]

[1] Paklihawa Campus, Institute of Agriculture and Animal Science, Tribhuvan University, Rupandehi 32900, Nepal; sirjan.bastola1@pakc.tu.edu.np (S.B.); ksavlamsal@gmail.com (K.L.); krishnakaphledr@gmail.com (K.K.); shambhu.shah@pakc.tu.edu.np (S.S.)
[2] Department of Agriculture, University of Arkansas at Pine Bluff, Pine Bluff, AR 71601, USA; shrestp0225@uapb.edu
[3] Department of Poultry Science, University of Georgia, Athens, GA 30602, USA
* Correspondence: anilg6623@gmail.com (A.G.); d.subedi@uga.edu (D.S.)

Simple Summary: *Escherichia coli* in raw milk poses economic losses and health risks due to antimicrobial resistance. In Siddarthanagar Municipality, Rupandehi, Nepal, 29.4% of buffalo milk samples were contaminated with *E. coli*. High resistance was observed against ceftriaxone and ceftazidime (100%), cotrimoxazole (86.7%), and amikacin (80%). Additionally, 86.7% of isolates were multidrug resistant. Although associations with risk factors lacked statistical significance, udder wash with antiseptics reduced *E. coli* contamination in milk, and detergent use during utensil washing showed promising trends. Farmer awareness of milk pasteurization and implementing food safety practices are crucial.

Abstract: *Escherichia coli* in untreated milk leads to economic losses from subclinical mastitis and reduced milk production, while also posing a public health risk due to the emergence of antimicrobial resistant strains, particularly associated with consuming unpasteurized milk and dairy products. This study aimed to determine the prevalence and antimicrobial resistance (AMR) of *E. coli* isolated from buffalo milk in Siddarthanagar Municipality of Rupandehi district, Nepal. A total of 102 milk samples were collected from lactating buffaloes. The isolation and identification of *E. coli* were carried out using enrichment media, selective media, and biochemical tests. Antimicrobial susceptibility testing was carried out using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (Merck), according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. Resistance was tested against gentamicin, amikacin, ciprofloxacin, enrofloxacin, ceftriaxone, ceftazidime, cotrimoxazole, and chloramphenicol. In addition to this, farmers were administered a questionnaire consisting of both open- and close-ended questions to identify various animal-related and management-related risk factors associated with the prevalence of *E. coli*. The prevalence of *E. coli* in our study was 29.4% (n = 30/102). Ceftriaxone and ceftazidime showed 100% resistance, while cotrimoxazole and amikacin showed 86.7% and 80% resistance, respectively. Furthermore, 86.7% of *E. coli* isolates were multidrug resistant (MDR). Despite suggestive trends, associations between *E. coli* prevalence and risk factors lacked statistical significance, necessitating further research. While some antibiotics exhibited effectiveness, many faced resistance, highlighting the need for prudent antimicrobial usage and increased awareness among farmers. Raising awareness about milk pasteurization and implementing food safety practices is essential for ensuring farmers and public health.

Keywords: antimicrobial resistance (AMR); dairy buffaloes; *Escherichia coli*; milk; Nepal

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1. Introduction

Nepal is a landlocked country in South Asia with a population of about 30 million. Agriculture is the main source of livelihood for most Nepalese, contributing about 27% of the gross domestic product (GDP) in 2019/20 [1]. Among the livestock species, buffalo and cattle are the most important for milk production, meat, and draught power. According to the Ministry of Agriculture and Livestock Development (MoALD), there were 7.5 million cattle and 5.3 million buffalo in Nepal in 2019/20 [1]. The total milk production was 2.3 million tons, of which 61% came from buffalo and 39% from cattle [1]. Buffalo milk has higher fat and protein content than cow milk, and is preferred more by consumers and processors [2,3]. In Nepal, buffalo are reared for milk, meat, draft power, and manure, and they are an important source of nutrition and income for many small-scale farmers [4].

Antibiotics are drugs that can kill or inhibit the growth of bacteria which cause infections. However, the misuse of antibiotics in human and animal health can lead to the emergence and spread of antibiotic resistance (AMR), which is the ability of bacteria to survive or grow in the presence of antibiotics [5]. AMR is a serious threat to public health, as it can make infections harder to treat and increase the risk of complications and death. AMR can compromise the health and productivity of animals and pose a threat to human health through foodborne and zoonotic infections [6]. Currently, at least 700,000 people worldwide die each year due to AMR. Without new and better treatments, the World Health Organization (WHO) predicts that this number could rise to 10 million by 2050, highlighting a health concern not of secondary importance [7]. In Nepal, antibiotics are widely used in livestock farming for various purposes, such as treating diseases, preventing infections, and promoting growth. However, there is a lack of regulation and monitoring of antibiotic use and resistance in the animal sector, which can contribute to developing and disseminating resistant bacteria among animals, humans, and the environment [8].

A study conducted in 2020 found that 16.5% of dairy cattle and buffalo in the western Chitwan region of Nepal were contaminated with multidrug-resistant *E. coli* [9]. In Nepal, the knowledge and awareness of farmers about AMR is low, and they have poor practice towards the cautious use of antibiotics despite having good knowledge [8,10,11]. *E. coli* is a common bacteria found in the gut of animals that can cause various infections in humans and animals. It is one such bacteria that can easily obtain genes that encode for antimicrobial resistance [12]. For these reasons, *E. coli* is commonly used as an indicator of antimicrobial resistance [13,14]. In addition, due to ubiquitous and commensal nature, *E. coli* also serves as a reservoir of antimicrobial resistance genes [15]. Over the years, multidrug-resistant (MDR) *E. coli* have been isolated from milk [16–18]. These MDR organisms pose public health threats if the milk is consumed unpasteurized [16], and in Nepal, the consumption and sale of raw milk to the public is common. Furthermore, they can contaminate milk products such as cheese. The use and misuse of antibiotics in livestock farming can contribute to the emergence and dissemination of antibiotic resistance among *E. coli* and other bacteria. Therefore, monitoring the prevalence and patterns of antibiotic resistance among *E. coli* is important. Hence, we aimed to determine the prevalence and AMR pattern of *E. coli* in milk from small-scale dairy buffalo in Siddarthanagar, Rupandehi, Nepal.

2. Materials and Methods

2.1. Study Area

The study was conducted in the southern part of Siddarthanagar municipality (27.5065° N, 83.4377° E) of the Rupandehi district. The laboratory work was conducted at the Veterinary Microbiology Laboratory of the Institute of Agriculture and Animal Science, Paklihawa Campus, Tribhuvan University.

2.2. Study Design

A cross-sectional study was conducted from July to September 2021. Milk samples from buffalo were collected aseptically. A structured questionnaire (Supplementary File) was used to assess the practices of buffalo farmers that could be risk factors for *E. coli*

contamination in milk, which may develop antibiotic resistance. The questionnaire was administered as a one-on-one interview in the local native language (Tharu) and Nepali to 79 farmers whose animals were sampled. The purpose of the survey was explained to the farmers, and their consent was obtained. The questionnaire was divided into three sections. The first section focused on the farmer's demographics, and the second section extracted information on the characteristics of their buffalo. The final section assessed the practices associated with the management and hygiene of buffalo. Characteristics of buffalo include breed, age, previous history of mastitis, history of any antimicrobials used, teat injuries, any disease condition, stage of parity, stage of lactation, milk yield per day, and frequency of milking. Practices associated with the management and hygiene of buffalo included the nature of housing, floor condition, feeding practices, type of milking, hand washing before and after milking, udder washing before and after milking, barn cleaning, type of utensils used for milking, use of antiseptics for udder wash and detergent for milking utensils, the practice of grazing, practice of teat dipping, dung management, treatment of buffalo while they are sick, and sufficient exposure of sunlight for buffalo.

2.3. Sample Size, Sampling Method, and Transportation

Purposive sampling was conducted to collect 102 milk samples from 79 small-scale buffalo farmers of Siddarthanagar. Farmers were instructed to clean udder and teats with water and dry them before sampling. The first few streaks of milk were discarded and about 10 mL of milk from every quarter was collected in new autoclaved plastic bottles. Animals currently under any antibiotic treatment were excluded from this study. The milk samples were examined for alterations in color, odor, and consistency. Samples exhibiting clots, flakes, blood, or any other noticeable changes in milk consistency or udder (indicators of clinical mastitis) were excluded [19]. Samples in a sterile universal sampling bottle were stored in the icebox and transported to the Veterinary Microbiology Laboratory of the Institute of Agriculture and Animal Science, Paklihawa Campus, Tribhuvan University, as soon as possible for further analysis and bacterial identification.

2.4. Bacteriological Isolation and Identification

Milk samples were used for the isolation and identification of *E. coli*. In total, 1 mL of each warmed (25.0 °C) sample was enriched in 10 mL of autoclaved nutrient broth (M002-500G, HiMedia, Thane, India), which was prepared in accordance with the manufacturer's Samples were incubated aerobically at 37.0 °C for 24.0 h. A loopful (10 µL) of the overnight culture was streaked on the eosin methylene blue (EMB) agar plates (M317-500G, HiMedia, India). The Petri dishes were incubated aerobically at 37.0 °C for 48.0 h. A green metallic sheen with typical violet colonies with foci was identified as presumptive *E. coli*. These colonies were selected and sub-cultured on EMB agar again and incubated at 37.0 °C for 24 h.

2.5. Biochemical Tests

Presumptive isolated colonies of *E. coli* were confirmed by gram staining and biochemical tests. Primary biochemical tests, i.e., an oxidase test, urease test, and catalase test, and secondary biological tests, i.e., Indole, Methyl Red, Voges–Proskauer, Citrate test, and Triple Sugar Iron test, were performed for confirmation (Table 1).

Table 1. Biochemical properties of *E. coli.*

Biochemical Tests	Properties of *E. coli*
Oxidase Test	Negative
Urease Test	Negative
Catalase Test	Positive
Indole Test	Positive
Methyl Red Test	Positive
Voges-Proskauer Test	Negative
Citrate Test	Negative
Triple Sugar Iron Test	Acid/Acid, Gas positive, no H_2S produced

2.6. Antibiotic Susceptibility Test (AST)

The Kirby–Bauer disk diffusion method was employed to test the susceptibility of isolates to a panel of antibiotics. The antibiotic disks used for *E. coli* were Gentamicin (10 µg), Amikacin (30 µg), Ciprofloxacin (5 µg), Enrofloxacin (5 µg), Ceftriaxone (30 µg), Ceftazidime (30 µg), Ceftazidime/Clavulanic acid (30/10 µg), and Cotrimoxazole (30 µg). These antibiotics were selected because they were the most readily available used and prescribed antibiotics in Nepal [20,21]. Bacterial colonies were swirled in distilled water and measured optical density (OD) of 0.15 and a transmittance percentage of 75.0% to attain a turbidity equivalent to 0.5 McFarland Standard (R092-1NO, HiMedia, India) using a Smart Digital Photo Colorimeter (UNILAB, Mumbai, India). This was poured onto an autoclaved Muller Hinton Agar (M173-500G, HiMedia, India), and antibiotic disks were placed 25.0 mm apart using sterile forceps. Plates were incubated overnight at 37.0 °C. The zone of inhibition was measured and interpreted based on the epidemiological cut-off values established by the European Committee on Antimicrobial Susceptibility Testing [22].

2.7. Identification of Multi-Drug Resistance (MDR)

MDR was defined as the resistance to more than two classes of antibiotics among all the antibiotics tested [23,24].

2.8. Multiple Antibiotic Resistance (MAR) Index Calculation

MAR index was determined and analyzed as per Krumperman's approach (1983), employing the following formula: a divided by b, where 'a' stands for the number of antibiotics to which an isolate displayed resistance, and 'b' represents the total number of antibiotics examined [25].

2.9. Data Analysis

Data were summarized using Microsoft Excel 2010 and analyzed utilizing the Minitab Rx64. The descriptive statistics were summarized using frequency and proportions. The chi-square test was used to test for association between independent variables (animal and management factors) and outcome variables (isolated *E. coli* from milk sample) at a 95% confidence interval with significant variables ($p < 0.05$) subjected to univariate logistic regression analysis.

3. Results

3.1. Characteristics of Sampled Animals

To determine the prevalence of *E. coli* in Siddarthanagar Rupandehi, a total of 102 buffalo were sampled from 79 households. Of those lactating buffalo, the number of Murrah cross was higher (66.7%, n = 68/102) than that of the Terai breed (33.3%, n = 34/102) (Table 2). Most of the buffalo sampled (78.4%, n = 80/102) were multiparous, and 57.8% (n = 59/102) were older than five years. Half of the buffalo (50%, n = 51/102) were in early lactation (1–3 months postpartum). The average milk yield per day per buffalo was 5.1 L, and 60.8% (n = 62/102) produced more than five liters. Milking practice in most of the buffalo (66.7%, n = 68/102) was twice a day, and fewer (33.3%, n = 34/102)

practiced milking once a day. All 79 households practiced hand-milking after hand and udder washing, whereas none of them practiced teat dipping.

Table 2. Descriptive characteristics of sampled buffalo at Siddarthanagar, Rupandehi (n = 102).

Variables	Number of Buffaloes (%)
Breeds	
Murrah Cross	68 (66.7%)
Terai	34 (33.3%)
Age (years)	
Less than or equal to five	43 (42.2%)
More than five	59 (57.8%)
Parity Stage	
Multiparous	80 (78.4%)
Uniparous	22 (21.6%)
Lactation Stage	
One to three months (Early)	51 (50%)
More than three months (Late)	51 (50%)
Milk Yield Per Day	
Less than or equal to five liter	62 (60.8%)
More than five liter	40 (39.2%)
Milking Practice	
Once a day	34 (33.3%)
Twice a day	68 (66.7%)

3.2. Descriptive Characteristics of Different Factors for Prevalence of E. coli

Some of the buffalo (18.6%, n = 19/102) had a previous history of mastitis, and 4.9% (n = 5/102) of them had teat injuries. Most of the animals (59.8%, n = 61/102) were raised under a semi-intensive production system, and 68.6% (n = 70/102) barns were with a concrete condition where barn cleaning practice was mostly (74.5%, n = 76/102) found to be twice a day. Very few farmers (5.9%, n = 6/102) used antiseptics for udder cleaning, whereas most of them (79.4%, n = 81/102) used detergent for milking utensils washing. Most farmers (94.1%, n = 91/102) used steel utensils for milking, and the remaining used plastic buckets. Grazing practice was found in 81.4% (n = 83/102) of buffalo, and dung shade distance was mostly (72.5%, n = 74/102) found less than three meters from the buffalo shed. In 23.5% (n = 24/102) of barns, sunlight exposure was found to be insufficient.

3.3. Prevalence of E. coli

Among 102 samples collected from 79 farmers, the prevalence of *E. coli* in buffalo milk was 29.4% (n = 30/102), and 70.6% (n = 72/102) were *E. coli* negative. Samples were considered positive from the results of eosin methylene blue (EMB) agar plates (Figure S1) and the different biochemical tests that were conducted (Table 1, Figure S2).

3.4. Management Factors for Prevalence of E. coli in Buffalo's Milk

Milk of buffaloes in which utensils used for milking were washed without detergent was 2.13× (95% CI: 0.69–6.44; p = 0.13) more likely to have a prevalence of *E. coli* than animals in which utensils used for milking were washed with detergent. As expected, buffaloes in which antiseptics were not used in the udder wash after milking were 2.15× (95% CI: 0.23–105.79; p = 0.48) more likely to have a prevalence of *E. coli* than those in which antiseptics were used in the udder after milking. Similarly, buffaloes near dung shade were 1.35× (95% CI: 0.47–4.30; p = 0.55) more likely to have a prevalence of *E. coli*

than animals far from dung shade. None of the animal and environmental-related factors were significantly associated with *E. coli* prevalence (Tables 3 and 4).

Table 3. Univariate analysis of animal factors associated with the prevalence of *Escherichia coli* in buffalo milk from Siddarthanagar, Rupandehi ($p \leq 0.05$ was considered statistically significant).

Animal Factors	Categories	Number of Animals (x)	Number of Positive (y)	Prevalence of *E. coli* (y/x)	Odds Ratio (95% CI)	*p*-Value
Age	>5 years	59	16	27.1%	0.77 (0.30–1.20)	0.55
	≤5 years	43	14	32.6%		
Breed	Murrah cross	68	17	25.0%	0.51 (0.19–1.36)	0.13
	Terai	34	13	38.2%		
California Mastitis Test (CMT)	Positive	27	8	29.6%	1.01 (0.33–2.89)	0.98
	Negative	75	22	29.3%		
Disease condition	Present	9	3	33.3%	1.22 (0.18–6.22)	0.12
	Absent	93	27	29.0%		
Lactation stage	>3 months	51	14	27.5%	0.83 (0.32–2.12)	0.66
	≤3 months	51	16	31.4%		
Mastitis history	Yes	19	8	42.1%	2.00 (0.61–6.32)	0.18
	No	83	22	26.5%		
Milk yield per day	>5 L	40	12	30.0%	1.05 (0.40–2.71)	0.92
	≤5 L	62	18	29.0%		
Parity stage	Multiparous	80	21	26.2%	0.52 (0.17–1.59)	0.18
	Uniparous	22	9	40.9%		
Teat injuries	Present	5	3	60.0%	3.83 (0.41–48.20)	0.12
	Absent	97	27	27.8%		
Milking practice	Once a day	34	9	26.5%	0.81 (0.28–2.18)	0.64
	Twice a day	68	21	30.9%		

Table 4. Univariate analysis of environmental factors associated with the prevalence of *Escherichia coli* in buffalo's milk Siddarthanagar, Rupandehi ($p \leq 0.05$ was considered statistically significant).

Environmental Factors	Categories	No. of Animals (x)	No. of Positive (y)	Prevalence of *E. coli* (y/x)	Odds Ratio (95% CI)	*p*-Value
Antiseptics washing during milking	No	96	29	30.2%	2.15 (0.23–0.59)	0.48
	Yes	6	1	16.7%		
Barn cleaning	Once a day	26	6	23.1%	0.65 (0.19–1.97)	0.65
	Twice a day	76	24	31.6%		
Detergent used	No	21	9	42.8%	2.13 (0.69–6.44)	0.13
	Yes	81	21	25.9%		
Drainage of water in the barn	No	20	5	25.0%	0.76 (0.19–2.53)	0.63
	Yes	82	25	30.5%		
Dung distance	<3 m	74	23	31.1%	1.35 (0.47–4.30)	0.55
	≥3 m	28	7	25.0%		
Floor condition	Mud	32	7	28.9%	0.58 (0.18–1.64)	0.26
	Concrete	70	23	32.9%		
Housing	Intensive	41	11	26.9%	0.81 (0.30–2.11)	0.64
	Semi-intensive	61	19	31.1%		
Milking utensils	Plastic	6	2	33.3%	1.21 (0.10–9.02)	0.82
	Steel	96	28	29.2%		

Table 4. *Cont.*

Environmental Factors	Categories	No. of Animals (x)	No. of Positive (y)	Prevalence of *E. coli* (y/x)	Odds Ratio (95% CI)	*p*-Value
Out grazing	Yes	83	24	28.9%	0.88 (0.27–3.17)	0.82
	No	19	6	31.6%		
Sunlight exposure	Insufficient	24	6	25.0%	0.75 (0.22–2.30)	0.59
	Sufficient	78	24	30.8%		

3.5. Antibiotic Susceptibility Test (AST)

Among eight antibiotics tested, the most resistance was seen with ceftriaxone (100%) and ceftazidime (100%), followed by cotrimoxazole (86.7%), amikacin (80.0%), and chloramphenicol (60.0%) (Table 5).

Table 5. Antibiotic susceptibility of *Escherichia coli* isolated from the milk of buffalo.

Antibiotics Used	Susceptible (%)	Intermediate (%)	Resistant (%)
Gentamycin (GEN 10)	60.0 (18/30)	13.3 (4/30)	26.7 (8/30)
Amikacin (AK 30)	6.7 (2/30)	13.3 (4/30)	80.0 (24/30)
Ciprofloxacin (CIP 5)	16.7 (5/30)	26.7 (8/30)	56.6 (17/30)
Enrofloxacin (Ex 5)	43.3 (13/30)	26.7 (8/30)	30.0 (9/30)
Ceftriaxone (CTR 30)	0.0 (0/30)	0.0 (0/30)	100.0 (30/30)
Ceftazidime (CAZ 30)	0.0 (0/30)	0.0 (0/30)	100.0 (30/30)
Cotrimoxazole (COT 25)	0.0 (0/30)	13.3 (4/30)	86.7 (26/30)
Chloramphenicol (C 30)	3.3 (1/30)	36.7 (11/30)	60.0 (18/30)

3.6. Multiple Antibiotic Resistance (MAR) Index of E. coli Isolates

Among 30 *E. coli*-positive isolates, the maximum number of bacteria, i.e., 9 (30.0%), were found to be resistant to 6 antibiotics with a MAR index of 0.8. The number of isolates that were resistant to the maximum number (seven) antibiotics was eight (26.7%), with a MAR index of 0.9 (Table 6).

Table 6. Multiple Antibiotic Resistance (MAR) index profile of *Escherichia coli* isolated from buffalo milk.

The Total Number of Antibiotics Used	Number of Antibiotic-Resistant	MAR Index	No. of Isolates
8	1	0.1	0
	2	0.3	0
	3	0.4	4
	4	0.5	5
	5	0.6	4
	6	0.8	9
	7	0.9	8

3.7. Multidrug-Resistant (MDR) Phenotype of E. coli Isolates

Among 30 *E. coli* isolates tested for five antibiotics groups, 86.7% were resistant to more than two antibiotics groups and were classified as MDR positive (Table 7, Figure 1).

Table 7. Multidrug-resistant (MDR) character of *Escherichia coli* isolated from buffalo milk.

The Total Number of Antibiotic Groups Used	Number of Antibiotics Group Resistant	Number of Isolates Resistant	Percentage (%) of Isolates Resistant	MDR
5	0	0	0.0	–
	1	0	0.0	–
	2	4	13.3	–
	3	5	16.7	+
	4	10	33.3	+
	5	11	36.7	+

	Aminoglycosides		Fluoroquinolones		Cephalosporins		Sulphonamides	Phenicols	MAR Index	MD
E. coli	GEN 10	AK 30	CIP 5	EX 5	CTR 30	CAZ 30	COT 25	C 30		
2b	R	S	R	R	R	R	R	R	0.88	+
3	S	R	R	R	R	R	R	R	0.88	+
4b	S	R	I	R	R	R	R	R	0.75	+
5	R	R	R	I	R	R	R	R	0.88	+
6b	R	R	R	S	R	R	R	R	0.88	+
22	R	R	R	S	R	R	R	I	0.75	-
25	R	R	R	R	R	R	R	I	0.88	-
27	R	R	R	I	R	R	R	I	0.75	-
28	I	R	R	R	R	R	I	R	0.75	-
30	I	R	R	I	R	R	R	I	0.63	-
32b	S	R	I	S	R	R	R	R	0.63	-
35	S	I	S	S	R	R	I	R	0.38	-
39a	S	I	S	S	R	R	R	I	0.38	-
39b	S	I	R	I	R	R	R	I	0.50	-
43	S	R	R	S	R	R	R	R	0.75	+
47	S	R	S	S	R	R	I	R	0.50	-
49	S	R	S	S	R	R	R	R	0.63	-
50	S	R	I	R	R	R	R	R	0.75	+
54	I	R	R	R	R	R	R	R	0.88	+
56	R	R	R	I	R	R	R	I	0.75	-
57	S	R	R	R	R	R	R	R	0.88	+
61	S	R	R	I	R	R	R	I	0.63	-
63a	R	R	I	S	R	R	R	R	0.75	-
68a	S	R	R	R	R	R	R	R	0.88	+
71	S	I	I	I	R	R	R	R	0.50	-
72	S	R	I	I	R	R	R	I	0.50	-
73	S	S	I	S	R	R	R	I	0.38	-
74a	S	R	I	S	R	R	I	I	0.38	-
76	I	R	S	S	R	R	R	S	0.50	-
79	S	R	R	S	R	R	R	R	0.75	+

Figure 1. Antibiotic resistance profile, Multiple Antibiotic Resistance (MAR) index, and Multidrug-resistant (MDR) character of *Escherichia coli* isolated from buffalo milk. R: Resistance, I: Intermediate Susceptible, S: Susceptible. GEN: Gentamicin; AK: Amikacin; CIP: Ciprofloxacin; EX: Enrofloxacin; CTR: Ceftriaxone; CAZ: Ceftazidime; COT: Cotrimoxazole; C: Chloramphenicol.

4. Discussion

E. coli is one of the major microbes found in the milk of buffalo as a bacterial load that may persist for a long period, directly affecting the health and milk yield of buffalo [3], and also poses a threat to public health [26]. In this study, the prevalence of *E. coli* in buffalo milk was found to be 29.4% (n = 30/102), which is consistent with previous findings reported by Khanal and Pandit (2013) [27], Verma et al. (2018) [28], and Acharya et al. (2017) [29]. These studies reported prevalence rates of 32.7%, and 21.3%, and 20.0%, respectively. Prevalence in our study was lower than the prevalence rates reported by Limbu et al. (2020) [30], Phattepuri et al. (2020) [31], and Jindal et al. (2021) [32], which were 55.0%, 60.0%, and 60.0% respectively. However, the prevalence in this study was higher than that reported by Singh et al. (2018) [33], Dhungana et al. (2011) [34], and Sharma et al. (2018) [35], which were 17.2%, 14.1%, and 8.4%, respectively. The variation in results may be attributed to individual animal characteristics such as age, health status, stress level, parity, and milk yield, as well as management, hygienic, and biosecurity factors. Unhygienic milking and handling practices employed by farmers have the potential to introduce *E. coli* contamination into raw milk, thereby presenting health risks to consumers [36].

Escherichia coli O157:H7 and other enterohemorrhagic *E. coli* (EHEC) represent food- and waterborne zoonotic agents capable of inducing diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in humans. These pathogens typically manifesting negligible or inconspicuous clinical manifestations within their animal reservoir [37]. Studies have suggested that temperature deviations during the transportation and handling of milk can lead to significant growth of *E. coli* O157:H7 [38,39]. There is a practice of consuming raw milk in Nepal, and temperature deviation of milk during handling, transportation, and storage is not uncommon. These scenarios and the occurrence of *E. coli* in buffalo milk, as shown by the study, highlight the zoonotic potential of *E. coli* O157:H7 and or EHEC. Thus, it is important to increase awareness and education on milk pasteurization and foodborne illness associated with milk to prevent the zoonotic transmission of this pathogen.

Our study revealed that *E. coli* isolates exhibited 100% resistance to ceftazidime and ceftriaxone, which is higher than the resistance rates reported in previous studies conducted by Elbehiry et al. (2021). They reported resistance rates of 66.7% for ceftazidime and 54.6% for ceftriaxone. Acharya et al. (2017) reported 100% susceptibility of *E. coli* isolates toward Ceftazidime, and Singh et al. (2018) reported 81.5% susceptibility of *E. coli* toward Ceftriaxone, which is not in agreement with the result of our study. Our study found that 60.0% of *E. coli* isolates were resistant to Chloramphenicol, which was more than the 25.9% reported by Singh et al. (2018). This dissimilarity may be due to the use and availability of different antibiotics in different locations and the frequency of their use. High levels of antibiotic resistance are likely attributed to the improper usage of antibiotics for disease treatment and the irrational application of antimicrobials for both therapeutic and prophylactic purposes. Additionally, the widespread availability and over-the-counter sale of these antimicrobials in the country exacerbates the challenge of curtailing their indiscriminate use [10].

Bhandari et al. (2021) found that 34.7% of *E. coli* isolates were resistant against ciprofloxacin, which is lower than in our study (56.67%). In our study, 86.7% of the *E. coli* isolates were found to be multidrug resistant (MDR). This finding aligns with Yadav et al. (2023), who reported a MDR prevalence of 78% among *E. coli* isolated from bovine milk [40]. Similarly, Bhandari et al. (2021) reported some of their isolated *E. coli* from cases of subclinical mastitis in lactating cows were found to be MDR. The emergence of ciprofloxacin resistance is particularly alarming on a global scale, given its classification as a critical drug for combatting drug-resistant pathogens in humans [41]. Moreover, 100% resistance to ceftazidime and ceftriaxone, third-generation cephalosporin attributable to the production of extended-spectrum β-lactamases, represents another clinically significant resistance mechanism in both human and animal health. Reports indicate a growing trend in resistance to fluoroquinolones and third-generation cephalosporins worldwide [42]. The

presence of such resistant bacteria in milk in Nepal, where unpasteurized milk consumption is prevalent and sales are not regulated, poses a substantial public health risk.

The MAR index is determined by comparing the number of antibiotics a bacterium is resistant to with the total number of antibiotics it encounters. A MAR index of 0.2 or higher in bacteria indicates exposure to multiple antibiotics, and suggests a high-risk contamination source. In our study, the MAR index of *E. coli* ranged from 0.38 to 0.88. A study in Uttar Pradesh India found a MAR index range of 0.29 to 0.71 [40]. A high MAR index in samples indicates widespread antibiotic resistance among bacteria. This poses a significant public health threat due to the potential for the spread of antibiotic resistance genes to humans via the consumption of unpasteurized milk.

The ubiquitous nature of *E. coli*, a Gram-negative bacterium found in farm environments, underscores the importance of barn cleaning and disinfection. These practices mitigate the risk of transmitting environmental pathogens to animals, thereby reducing the likelihood of infection. Milk collected from buffalo where antiseptics were used for udder wash was less likely to be contaminated with *E. coli*. Similarly, buffalo milk collected in utensils washed without detergent exhibited a trend towards higher prevalence of *E. coli* compared to those washed with detergents. Thus, it is crucial to give attention toward barn cleaning and disinfection, which small-scale dairy farmers often overlook.

Additional investigations involving cattle, larger sample sizes, and utilizing molecular techniques are recommended. In this research, we used eight antibiotics to test AMR. It is recommended to use a greater number of antibiotics in future studies. Given the observed resistance of up to 100% to critically important antibiotics, it is advised to consume milk only after thorough boiling or pasteurization.

5. Conclusions

Our study identified the presence of multidrug-resistant *Escherichia coli* (MDR *E. coli*) in the milk of small-scale dairy buffalo in the Rupandehi district of Nepal. The contamination of milk with *E. coli* is likely attributed to unhygienic milking practices and coupled with the resistance of critically important antibiotics, poses a significant public health risk to consumers of raw or unpasteurized milk. Urgent measures such as mandatory pasteurization of milk by farmers are necessary to address this issue. Farmers in this region require increased awareness and education on hygienic practices and antibiotic resistance to effectively mitigate the risk of contamination and multidrug-resistant pathogens in milk.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/zoonoticdis4030016/s1, Figure S1: *Escherichia coli* on eosin methylene blue EMB agar showing green metallic sheen colonies.; Figure S2: IMViC Test and Triple Sugar Irom (TSI) Test. A: IMViC test, the first image showing Indole, Methyl Red, Voges-Proskauer, and Citrate tubes before inoculation with *E. coli*. The second image shows tubes Indole and Methyl red positive, Voges-Proskauer and Citrate negative after inoculation of *E. coli* and incubation for 24 hours at 37 °C. B: TSI Slant test, first image showing TSI slant before inoculation with *E. coli,* second image showing growth of *E. coli* with yellow slant, yellow butt, gas production but no H2S production after 24-hour incubation at 37 °C.

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Informed Consent Statement: Signed consent was obtained from the farmers to participate in this study after explaining the objective of the study. No individual identifier was collected, and data were analyzed in a group to preserve the individual farmer's identity.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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