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RESEARCH ARTICLE

Trends in Frequency, Potential Risks and Antibiogram of *E. coli* Isolated from Semi-Intensive Dairy Systems

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ABSTRACT

Semi-intensive dairy systems are prevailing in tropical and subtropical countries that need to be probed for public health concern pathogens. The current study was designed to map trends in prevalence of dairy-originated *E. coli*, associated risk factors, and altering *in-vitro* drug susceptibility patterns. A total of n=200 subclinical mastitis milk samples were intended to be collected using purposive sampling method from semi-intensive dairy systems for which n=764 dairy animals (n=440 buffaloes, n=324 cattle) were screened. Standard biochemical and microbiological methods coupled with polymerase chain reaction (23sRNA) were applied to identify *E. coli* from subclinical milk samples. The study, overall, found a 26.18% (200/764) prevalence of subclinical mastitis and 13.50% (27/200) *E. coli* from subclinical samples. Among assumed risk factors, lack of use of teat dip (OR=8.26, C.I. = 2.73–24.91), higher age groups (OR=17.87, C.I. = 4.42–72.16), parity number >3 (OR=3.68, C.I. = 1.59–8.49), underweight animals (OR=2.89, C.I. = 1.11–7.53), and mid-lactation (OR=14.94, C.I. = 3.04–73.24) were dominant potential risk factors for *E. coli* infection. Antibiogram showed 42.86 and 21.43% of *E. coli* isolates resistant to amoxicillin-clavulanate and oxytetracycline, respectively. It was noted that more than 40% (42.86, 60.87, 57.89, 66.86, and 67.86%) of *E. coli* fall in intermediate susceptible cadre against 62.5% of tested antibiotics. In conclusion, increasing percentages of *E. coli*, higher number of potential risk factors, and antibiotic susceptibility inclining towards resistance demands stern compliance in anticipated time to avoid any grave situation.

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INTRODUCTION

It is evident fact that the dairy sector has tremendously grown in recent years while the semi-intensive system is prevailing. These systems contribute 21% of milk production in the Mexican dairy milk sector (Ramírez-Rivera *et al.*, 2019) while other parts of the world, predominantly Asian countries, it is increasing day by day. These commercial dairy systems are better than conventional while the concern of justified use of

antibiotics and hygiene remains a question (Swar *et al*., 2021; Abouelhag *et al*., 2021). Apart from their strength in contribution, these are either overlooked for prevalence and drug resistance of milk-borne pathogens. Regular epidemiology of pathogens along with their prior drug resistance against antibiotics strengthens control strategies. Pathogens are becoming multidrug-resistant (Sweeney *et al*., 2018) against FDA-approved drugs such as β-lactams, sulfonamides, quinolones, macrolides, and tetracyclines (Yu *et al*., 2020). The unjustified use of antibiotics in the dairy industry is compromising animal and public health in terms of widespread antimicrobial resistance (Anwar *et al*., 2020).

The most reported infectious agents potentially contaminating mammary glands and milk are *Escherichia coli*, *Streptococcus dysgalactiae*, *Klebsiella spp*, non-*Staphylococcus aureus* (NAS), and *Staphylococcus aureus* (Cheng *et al*., 2019; Aqib *et al.*, 2021; Khalaf *et al.*, 2021). The reports state 20-33% of *E. coli* isolated from raw milk samples become resistant to at least one antibiotic, and about 20% of isolates are resistant to two or more antibiotics (Martínez-Vázquez *et al*., 2018). *E. coli* is also considered a highly efficient reservoir of antibiotic-resistant genes that can be transferred to other micro-organisms (Hinthong *et al*., 2017). Such behavior is exhibited by an interplay of different drug resistance mechanisms which develops through the possession of external drug resistance factors such as carbapenems, quinolone resistance factors, extended beta-lactamases, and aminoglycoside modifying enzymes or through natural mutations (Cag *et al*., 2016). Some of the prominent resistant genes in *E. coli* include *strA, tetA, sulI, ampC, tetB, strB*, and *sulII* in the United States while *blaCTX-M*, *bla-TEM*, *tetA*, *tetB*, *strA*, and *strB* from Mexican dairy (Jiménez Mejía *et al*., 2017).

It has thus equally become important to consider mastitis-based *E. coli* as a major pathogen for regular epidemiology cum antibiotic susceptibilities to make a correct choice of therapeutic candidates (Yu *et al*., 2020). Alongside, repeated exposure to sub-lethal concentrations of antibacterial agents undoubtedly contributes to resistance development as semi-intensive dairy systems are frequent users of antibiotics without stern prescription by a veterinarian. The current study was designed to investigate and provide an updated record of the prevalence of *E. coli* of semi-intensive dairy systems, potential risk factors involved, and susceptibility pattern of these isolates to commonly used antibiotics.

MATERIALS AND METHODS

Sampling: Milk samples were collected from various farms located in and around district Faisalabad based on the accessibility of the dairy population. The study aimed to collect n=200 subclinical mastitis milk samples by using purposive sampling technique (Thrusfield *et al.*, 2018). For this purpose, we had to screen n=764 dairy animals (n=324 cattle, n=440 buffaloes) in our study. The samples were screened by Surf Field Mastitis Test (SFMT) to identify subclinical mastitis (Muhammad *et al*., 2010). Positive samples were transported to Mastitis Research Laboratory, Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad maintaining cold chain at 4°C. Furthermore, the questionnaire was filled out at the time of sampling having information related to animal species, udder condition, and consistency, physical characteristics of milk, teat dipping, age, lactation stage, hygienic conditions during milking for risk factor analysis associated with spread of mastitis caused by *E. coli* (Akter *et al.*, 2020).

Isolation and confirmation of *E. coli*: Positive samples were centrifuged at 6000×g for five minutes. The sediments were incubated in sterile nutrient broth for 24 hours at 37°C. Incubated broth was again centrifuged at 6000×g for five minutes. The sediments were swabbed on blood agar following the same incubation conditions. Further, culturing was done on differential media MacConkey agar (Fig. 2) for differentiation and isolation of *E. coli*. Microbiological and biochemical tests did confirmation of the *E. coli* strains following the guidelines of Bergey's Manual of Determinative Bacteriology (Bergey & Holt, 1994).

Molecular characterization of *E. coli*: *E. coli* was further identified targeting 23S rRNA gene using E23S-F: ATCAACCGAGATTCCCCCAGT; E23S-R: TCACTA TCGGTCAGTCAGGAG) primers at 231 bp product. The conditions used for this PCR were taken from (Shafiq *et al.*, 2021). The PCR product was finally run on 2% agarose gel electrophoresis.

***In-vitro* antibiotic susceptibility testing for MDR *E. coli*:** Fresh growth of *E. coli* was adjusted at 1.5×10^8 CFU/mL and swabbed on Mueller Hinton agar while various antibiotic discs such as chloramphenicol (30µg), ciprofloxacin (5µg), gentamicin (10µg), amoxicillin-clavulanate (20µg), levofloxacin (5µg), oxytetracycline (20µg), ampicillin (10µg), enoxacin (10 µg) were aseptically placed. Incubation was given at 37°C for 24 hours and zones of inhibition were measured and compared with guidelines of Clinical Laboratory and Standards Institute (CLSI, 2015).

Statistical Analysis: Prevalence was calculated by the formula described by (Thrusfield, 2018);

$$\text{Prevalence (\%)} = \frac{\text{No. of infected animals (n)}}{\text{Total number sampled (N)}} 100$$

The descriptive statistics were applied for estimation of *in-vitro* antibiotic susceptibility, while risk factor analysis was done by chi-square and odds ratio at 5% probability using SPSS version 22.

RESULTS

Percentage positive subclinical mastitis infection and *E. coli*: The present study found overall 26.18% (200/764) subclinical mastitis from the dairy origin with a higher percentage from cattle (14.53%, 111/764) than that of buffalo (11.65%, 89/764) based on overall screened (n=764) sample basis. Subclinical mastitis positive samples from cattle (calculated from total cattle screened, n=324) were found to be 34.26% (111/324). Percentage of buffaloes positive for subclinical mastitis (calculated from a total number of buffaloes screened, n=440) were noted to be 20.23% (89/440). In this case, a significant association of species (cattle & buffalo) was noted with subclinical mastitis. Percentage positive *E. coli* were

13.50% (27/200) from sub-clinical mastitis samples. The prevalence of *E. coli* in milk samples was found to be higher in subclinical mastitis milk (14.41%, 16/111) as compared to buffalo (12.36%, 11/89) while its association with species of dairy animals stood non-significant (P>0.05) (Fig. 1). PCR identified *E. coli* at 23s RNA gene-specific for *E. coli* to make sure about *E. coli* in the current study (Fig. 3).

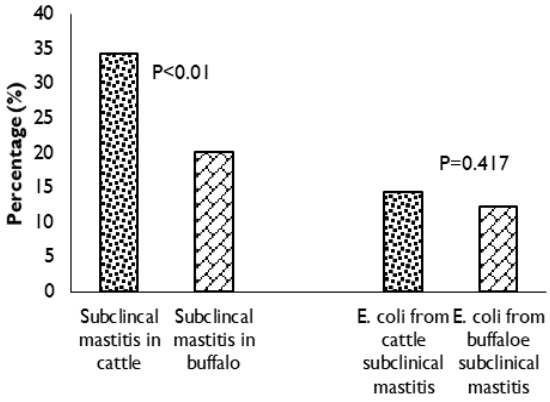

Fig. 1: Association of species (cattle and buffalo) with subclinical mastitis and *E. coli* (*p*-value<0.05 indicate significant association of species factor with the prevalence of subclinical mastitis and prevalence of *E. coli*, NB: Chi-square test was used).

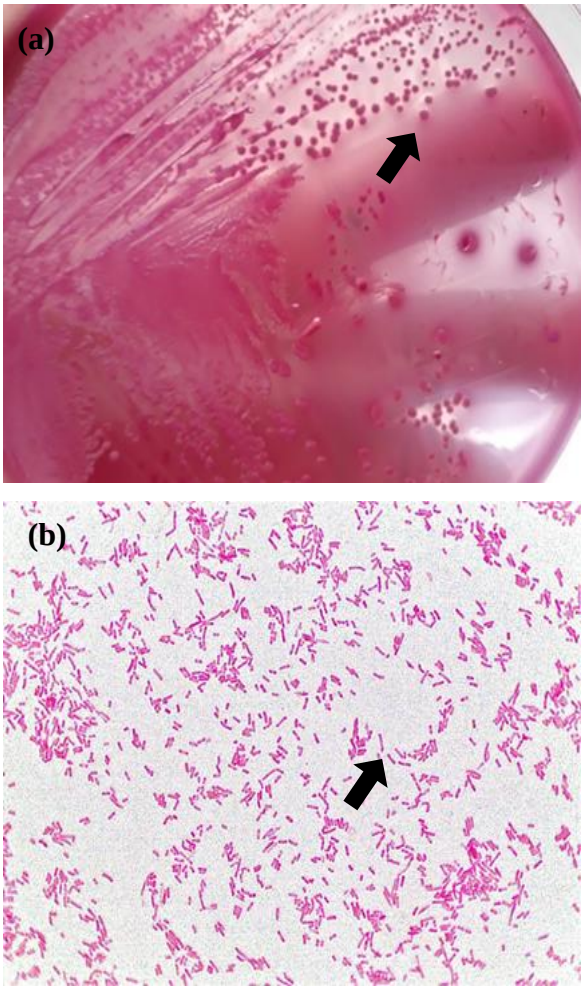

Fig. 2: Biochemical expression of *E. coli* on agar and gram staining. (a) arrow pointing pink colonies of *E. coli* on MacConkey agar (b) arrow pointing out pink colored (gram-negative color) short rods of *E. coli* (right) visualized at 100X magnification.

Risk factor analysis associated with *E. coli*: The regression analysis revealed fibrosed udder, milk having changed color, no teat dipping, age ≥5 years, parity number >3, mid & late lactation stage, and underweight animals as potential risk factors for acquisition of *E. coli* mastitis (OR> 1, P<0.05). Swollen udder, tick infestation,

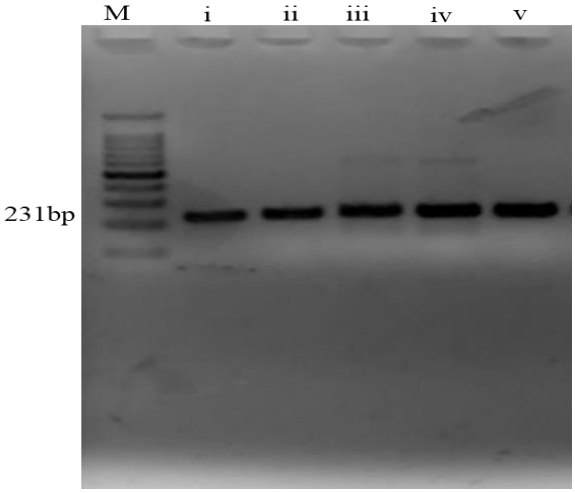

Fig. 3: PCR detection of 23s RNA based *E. coli* isolated from milk samples. M=marker, i, ii, iii, iv= Sample numbers, v=Positive control.

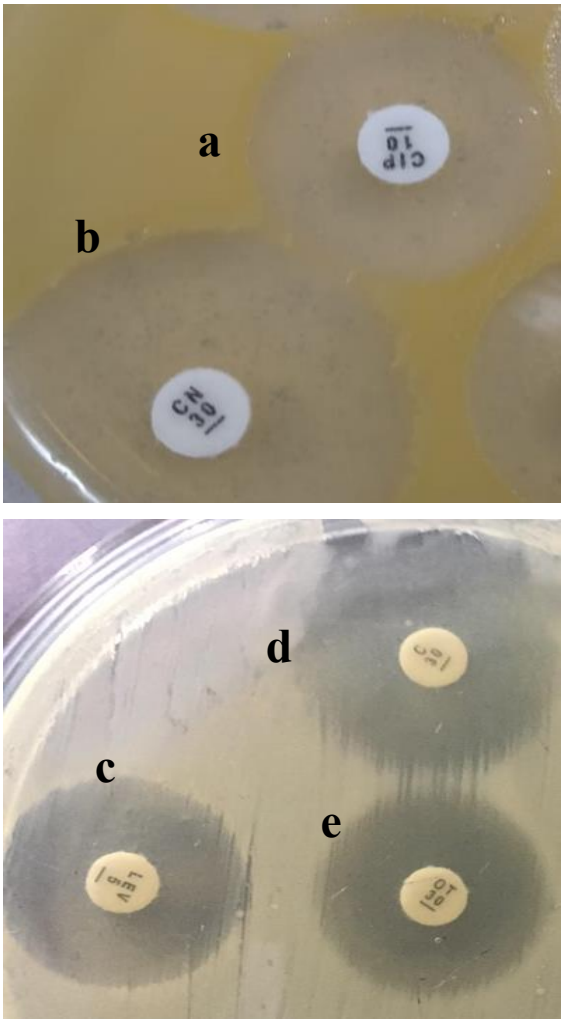

Fig. 4: Antibacterial expression of *E. coli* against different antibiotics (a=Ciprofloxacin, b=Gentamicin, c=Levofloxacin, d=Chloramphenicol, e=Oxytetracycline).

Table 1: Risk factors associated with the spread of *E. coli* among dairy animals

Parameters	Levels	Total	Positive	Percentage (%)	Odd Ratio	CI (95%)	P-value
Udder condition & consistency	Normal	183	21	11.48	1	-	-
	Swollen	13	04	30.77	3.4286	0.9701-12.1173	0.0558
	Fibrosed	04	02	50.00	7.7143	1.0315-57.6909	0.0466
Physical Milk characteristics	Normal	173	12	6.94	1	-	-
	Flakes	02	01	50.0	13.4167	0.7892-228.0872	0.0725
	Color changed	25	14	56.0	17.0758	6.3852-45.6654	< 0.0001
Teat dip	Yes	106	04	3.77	1	-	-
	No	94	23	24.47	8.2606	2.7383-24.9199	0.0002
Teat abnormality	Normal	174	21	12.07	1	-	-
	Stenosis	08	02	25.0	2.4286	0.4599-12.8246	0.2960
	Free milker	18	04	22.22	2.0816	0.6262-6.9194	0.2316
Age group (Years)	2-4	155	12	7.74	1	-	-
	5-8	35	09	25.71	4.1250	1.5794-10.7732	0.0038
	>8	10	06	60.0	17.8750	4.4274-72.1676	0.0001
Parity number	1-3	135	11	8.15	1	-	-
	>3	65	16	24.62	3.6809	1.5956-8.4913	0.0022
Lactation stage	Early	95	02	2.11	1	-	-
	Mid	37	09	24.32	14.9464	3.0498-73.2486	0.0009
	Late	68	16	23.53	14.3077	3.1649-64.6810	0.0005
Tick infestation	Yes	39	09	23.08	2.3833	0.9770-5.8138	0.0563
	No	161	18	11.18	1	-	-
Feeding	Underfed	45	07	15.56	1.2434	0.4891-3.1608	0.6472
	Proper	155	20	12.90	1	-	-
Body condition	Under weight	31	08	25.81	2.8913	1.1102-7.5300	0.0297
	Medium	149	16	10.74	1	-	-
	Overweight	20	03	15.0	1.4669	0.3870-5.5602	0.5730
Hygienic Conditions during milking	Yes	131	08	6.11	1	-	-
	No	69	19	27.54	1.0358	0.4257-2.5199	0.9382

P<0.05 indicate significant association; CI= Confidence interval at 95%.

Table 2: Antibiogram of *E. coli* against different antibiotics

Antibiotic	Resistant (%)	Intermediate (%)	Sensitive (%)
Amoxicillin–Clavulanate	42.86	42.86	14.28
Levofloxacin	7.14	14.28	78.58
Oxytetracycline	21.43	35.71	42.86
Chloramphenicol	13.04	60.87	26.09
Ampicillin	13.16	23.68	63.16
Gentamicin	13.16	57.89	28.95
Enoxacin	12.71	66.86	20.43
Ciprofloxacin	10.71	67.86	21.43

P<0.05 indicate a significant association.

Table 3: Average zone of inhibitions (mm) shown by different antibiotics against *E. coli*

Antibiotic	Resistant (Mean±Std)	Intermediate (Mean±Std)	Sensitive (Mean±Std)
Amoxicillin - Clavulanate	11.00±1.15	15.25±0.50	19.50±1.73
Levofloxacin	11.25±.45	17.50±0.41	24.50±2.08
Oxytetracycline	11.50±1.29	15.00±0.81	20.75±0.95
Chloramphenicol	12.00±1.41	18.25±1.50	25.25±2.63
Ampicillin	9.81±0.23	13.25±0.95	20.00±3.36
Gentamicin	10.75±1.50	15.25±1.50	19.25±0.96
Enoxacin	21.58±1.83	00.00	00.00
Ciprofloxacin	16.00±1.41	23.25±1.25	33.75±3.50

Std= Standard deviation.

underfed animals, unhygienic milking conditions, overweight animals and teat abnormality were noted to be important risk factors having (OR>1.00) but they were not significantly (P>0.05) associated with the spread of *E. coli*. The percentage of *E. coli* varies in different levels as listed in Table 1.

Shifts in patterns of antibiotic susceptibilities of *E. coli*: The *in-vitro* antibiotic susceptibility testing revealed 42.86 and 21.43% of *E. coli* resistance against amoxicillin-clavulanate and oxytetracycline while in case of levofloxacin, ampicillin, and oxytetracycline percentage sensitive isolates were recorded to be 78.58%, 63.16%, and 42.86%, respectively (Table 2, Fig. 4). Chloramphenicol, gentamicin, and ciprofloxacin presented >20% efficacy against *E. coli* isolated from dairy origin. An important phenomenon in this study was noticed that more than 40% (42.86, 60.87, 57.89, 66.86, and 67.86%) of *E. coli* isolates shifted their activity from sensitive to intermediate susceptibility cadre against more than 62.5% (5 out of 8) of antibiotics. Moreover, 12-24% of intermediate susceptibility was noted against 25% of antibiotics tested in this trial (Table 2). The *in-vitro* response of all antibiotics varied significantly (P<0.05) when compared amongst resistant, intermediate, and sensitive cadres observed, listed in Table 2. The mean zones (mm) expressed by different antibiotics in various categories of resistant, intermediate, and sensitive cadre are listed in Table 3.

DISCUSSION

The results of the current study regarding the percentage of positive *E. coli* from milk samples are in line with the findings of (Yu *et al.*, 2020) who reported 11.1% isolation of *E. coli* from mastitis milk samples. Similarly, the percentage of *E. coli* reported in different researches conducted in and around the current study area was 12.6% (Ali *et al.*, 2017), and 14.4% (Feng *et al.*, 2018). Subclinical mastitis status of bovine in the current study was in line with findings of (Baloch *et al.* 2016) who reported 26.95% subclinical mastitis. Contrary to the findings of the current study, 32.5 and 33% subclinical mastitis was reported by (Abebe *et al.*, 2016; Mekonnen *et al.*, 2017), respectively. Contrary to the findings of the current study from the same region, subclinical mastitis was noted to be 61.60% in conventional dairy systems of district Faisalabad (Naseer *et al.*, 2021). Similarly, in a recent study, 45.97% prevalence of subclinical mastitis was noted in the same district (Javed *et al.*, 2021). The difference in prevalence in the same region might be due to sample sources. At semi-intensive dairy systems,

hygienic measures are adopted that prevent the significant prevalence of mastitis.

Findings of analysis of risk factors like number of lactations, unattended animals, and udder/teat anomalies were in line with the study of (Firth *et al.,* 2019). The lactation stage, changes in milk, parity number, and farm management were strongly associated with mastitis occurrence and persistence (Song *et al*., 2020). Lack of post milking teat dipping, an ignorant attitude of farmers towards animal health, larger farms with a greater number of unattended animals, and compromised udder health was strongly increasing the risk of mastitis persistence in dairy farms (Deng *et al.,* 2019). Skin lesions on the teats or udder, increased age, parity, within-herd maintenance of pathogen carrier udders, and stage of lactation were associated with increased risk of mastitis (Seligsohn *et al.,* 2020). A study depicted the odds ratio to be significantly higher in poor body condition animals, animals in late lactation, and with teat anomalies (Akter *et al.,* 2020).

Antibiogram of *E. coli* isolates from mastitic milk samples was in line with findings of (Yu *et al.,* 2020) who found 25.3% *E. coli* resistant to amoxicillin, 30.1% resistant to ampicillin. Response to ciprofloxacin, gentamicin, chloramphenicol, and tetracycline found in their study was in line with our study. *E. coli* is also considered as the reservoir of many antibiotic resistance coding genes that can be transferred to other pathogens too. Furthermore, ESBL producing *E. coli* and various groups which are linked to *bla* genes such as ST-167, ST-410, blaCTX-M15, ST-10, blaCTX-M55, ST-23 complex, and blaCTX-M14 are responsible for the development of resistance in *E. coli* (Su *et al*., 2016). The presence of ESBL producing *E. coli* and treatment failure in milk animals have been documented in multiple studies due to acquisition of any of the resistance mechanisms, *viz*., inactivation or modification of antibiotic; change in the antibiotic target site; modification in the metabolic pathways to reduce antibiotic effect; and by reducing entry and/or activating active efflux of the antibiotic (Sharma *et al*., 2018; Mehmood *et al*., 2020; Du *et al*., 2022).

Conclusions: The study concluded an overall higher prevalence of subclinical mastitis and *E. coli* from these samples. Furthermore, most of the potential risk factors associated with *E. coli* noted in this study were the point of consideration. The *in-vitro* antibiotic testing revealed higher resistance against amoxicillin-clavulanate, while higher efficacy was presented by levofloxacin, oxytetracycline, and ampicillin. Particularly, intermediate susceptible isolates against more than sixty percent of antibiotics indicated emerging resistance in *E. coli.* The current study thus provides baseline information about semi-intensive dairy systems that require immediate attention for effective control measures to avoid antimicrobial resistance, save public health, and health optimization.

Authors contribution: MAA, KA, MAA and AIA conceived the idea; MAA and MAN did research work; AIA, IM, and MS analyzed data and wrote the initial draft; ZAB, AS, MAA, and HS did statistical analysis; TZ, AIA, SA, FK, and AM revised manuscript.

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