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Original article

Virulence determinants and antimicrobial resistance of *E. coli* isolated from bovine clinical mastitis in some selected dairy farms of Bangladesh

Md.Abdus Sattar Bag [a], Md.Shahidur Rahman Khan [a], Md.Deluar Hossain Sami [a], Ferdousi Begum [b], Md.Shafiqul Islam [a], Md. Mizanur Rahman [c], Md.Tanvir Rahman [a], Jayedul Hassan [a,*]

[a] *Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh*
[b] *Department of Livestock Services, Ministry of Fisheries & Livestock, Krishi Khamar Sarak, Farmgate, Dhaka 1215, Bangladesh*
[c] *Border Guard Bangladesh Farm, Head Quarter Battalion, Border Guard Bangladesh, Pilkhana, Dhaka 1205, Bangladesh*

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A B S T R A C T

E. coli is one of the major significant pathogens causing mastitis, the most complex and costly diseases in the dairy industry worldwide. Present study was undertaken to isolate, detect the virulence factors, phylogroup, antimicrobial susceptibility and antimicrobial resistance genes in *E. coli* from cows with clinical mastitis. A total of 68 milk samples comprising 53 from clinical mastitis and 15 from apparently healthy cattle were collected from four different established dairy farms in Bangladesh. *E. coli* was isolated from the milk samples and identified by PCR targeting *malB* gene and sequencing of 16S rRNA gene. *E. coli* isolates were screened by PCR for the detection of major virulence genes (*stx*, *eae* and *cdt*) of diarrheagenic *E. coli* followed by phylogenetic grouping. Antimicrobial susceptibility of the *E. coli* isolates was determined by disk diffusion test and *E. coli* showing resistance was further screened for the presence of antimicrobial resistance genes. *E. coli* was isolated from 35.8% of the mastitis milk samples but none from the apparently healthy cattle milk. All the *E. coli* isolates were negative for *stx*, *eae* and *cdt* genes and belonged to the phylogenetic groups A and B1 which comprising of commensal *E. coli*. Antibiotic sensitivity testing revealed 84.2% (16/19) of the isolates as multidrug resistant. Highest resistance was observed against amoxicillin (94.5%) followed by ampicillin (89.5%) and tetracycline (89.5%). *E. coli* were found resistant against all the classes of antimicrobials used at the farm level. Tetracycline resistance gene (*tetA*) was detected in 100% of the tetracycline resistant *E. coli* and *bla*TEM-1 was present in 38.9% of the *E. coli* isolates. Findings of this study indicate a potential threat of developing antimicrobial resistance in commensal *E. coli* and their association with clinical mastitis. Occurrence of multidrug resistant *E. coli* might be responsible for the failure of antibiotic therapies in clinical mastitis as well as pose potential threat of transmitting and development of antibiotic resistance in human.
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1. Introduction

Bovine mastitis is one of the most complex and costly diseases in the dairy industry due to its high prevalence and economic losses worldwide (Seegers et al., 2003). Mastitis was reported to attribute 1.5 – 2.0 million US$ economic losses every year in the USA (Sharma et al., 2012). In Bangladesh mastitis causes an economic losses equivalent to Taka 122.6 (USD 2.11) million every year through the reduction of milk production and deterioration of milk quality (Biswas et al., 2020).

Mastitis is caused by an array of microorganisms including virus, bacteria, mycoplasma and yeast with bacteria being the major pathogen associated with the onset of clinical form of the disease (Egwu et al., 1994; Rahman et al., 2013). *E. coli* is major etiology among the bacteria predominantly associated with bovine mastitis worldwide (Barkema et al., 1998; Gao et al., 2017; Lan et al., 2020; Mahbub-E-Elahi et al., 1996; Radostits et al., 2000; Tenhagen et al., 2009; Verbeke et al., 2014).

E. coli is a Gram negative, rod-shaped, facultative anaerobic bacterium. Pathogenic *E. coli* can be categorized based on serogroups, pathogenic mechanisms, variation in epidemiology and different

* Corresponding author at: Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh.
E-mail address: dr_jahid@bau.edu.bd (J. Hassan).
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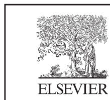

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interaction with the intestinal mucosa, clinical symptoms or virulence factors (Breland et al., 2017; Kaper et al., 2004).

Mastitis with *E. coli* varies from mild to very severe or even fatal (Shpigel et al., 2008; Wenz et al., 2001). *E. coli* associated with clinical mastitis possesses high genotypic variability and clinical severity varies among farms, groups and probable specific cow factors (Wenz et al., 2006). Most of the *E. coli* associated with clinical mastitis is typical commensals; however, pathogenic variants were also reported (Momtaz et al., 2012; Rangel and Marin, 2009; Suojala et al., 2011). Shigatoxigenic *E. coli* (STEC) are one of the pathogenic variants reported from clinical mastitis (Momtaz et al., 2012; Rangel and Marin, 2009). Several studies were performed to elucidate the virulence determinants and reported shigatoxin encoding genes (*stx1*, *stx2*) and *eae* being the most important virulence determinants in *E. coli* isolated from bovine mastitis (Güler and Gündüz, 2007; Kobori et al., 2004; Paton and Paton, 1998; Wenz et al., 2006). STEC are considered as the most important pathogens reported from food borne disease outbreaks in the recent years and are associated severe diseases in human including bloody diarrhea (Karmali, 1989; Nataro and Kaper, 1998; Pandey et al., 2003). Furthermore, they are often associated with life threatening disease outcomes such as hemolytic uremic syndrome (HUS) and Hemorrhagic colitis (HC) in human (Beutin et al., 2004; Karmali et al., 1983; Paton and Paton, 1998).

Antimicrobial therapy is practiced to control bovine mastitis. However, in most of the cases antimicrobial therapy does not follow prior susceptibility testing of the pathogens and thus misuses or suboptimal doses of the antimicrobials resulted in the emergence of antimicrobial resistant bacteria (Mia et al., 2017; Van Boeckel et al., 2015; Zhang et al., 2018). *E. coli* isolated from bovine mastitis were resistant to at least one of the antimicrobial classes (Fairbrother et al., 2015; Suojala et al., 2011). Moreover, multidrug resistant *E. coli* have been reported from bovine mastitis (Lan et al., 2020; Tahar et al., 2020). It has been reported that antimicrobial resistant bacteria cause more severe and persistent form of mastitis compared to those caused by antibiotic susceptible counterparts. Furthermore, occurrence of multidrug resistant virulent *E. coli* in bovine mastitis is a critical public health concern which threatens the public of transmitting zoonoses and food toxin infections (Blum et al., 2008; Erb et al., 2007; Fernandes et al., 2011; Johnson et al., 2008). Thus, a thorough study on the virulence determinants and antimicrobial resistance in *E. coli* associated with clinical mastitis is critical for the proper control of mastitis and protect human from the risk of getting infection from these pathogenic bacteria through consumption of contaminated milk.

Occurrence of virulence determinants and antimicrobial resistance in *E. coli* have been studied in different part of the world (Lan et al., 2020; Mora et al., 2005; Obaidat et al., 2018; Tark et al., 2017; Tavakoli and Pourtaghi, 2017; Zhao et al., 2018). However, research in Bangladesh is mostly focused on the risk factors or the subclinical form of the diseases (Islam et al., 2011, 2010; Rahman et al., 2009). Therefore, the present study was aimed at the isolation of *E. coli* from bovine clinical mastitis, assessing their virulence profiles, phylogenetic groups, antimicrobial susceptibility profile and presence of antimicrobial resistance genes.

2. Materials and methods

2.1. Sample collection

A total of 68 milk samples comprising 53 clinical mastitis and 15 apparently healthy cattle were collected from four prominent dairy farms in Bangladesh (Table 1). Farms containing more than 150 dairy cattle heads and history of persistent mastitis were purposively selected in this study. Milk samples were collected from all the mastitic cattle each farm through a single visit during the period from December 2019 to December 2020. Ten (10) ml of milk was aseptically collected directly from the udder of each cow in sterile falcon tube and carried to the laboratory in ice box. Information regarding the antimicrobials used to control mastitis and other disease conditions in the study farms were recorded during sample collection.

2.2. Enrichment and isolation of E. coli

Enrichment and isolation of *E. coli* from the milk samples were performed according to the protocol described by Fahim et al., 2019 with slight modification. Five hundred microliter (500 µl) of the collected milk sample was inoculated into 4.5 ml Luria Bertani (LB) broth (HiMedia, India) followed by incubation overnight at 37 °C. 100 µl of the enriched culture was spread onto Eosin Methylene Blue Levine agar (Liofilchem, Italy) and incubated overnight at 37 °C. After overnight incubation colonies with greenish metallic sheen were picked and purified colonies were isolated by subsequent streaking onto EMB agar plates (Liofilchem, Italy).

2.3. Genomic DNA extraction

Total bacterial genomic DNA was extracted by boiling method following the protocol describe earlier (Hassan et al., 2019) with slight modifications. Briefly, a single colony of the bacteria was cultured overnight at 37 °C into 3.0 ml LB broth. Bacteria were collected from 1.0 ml of the overnight culture by centrifugation at 10000 rpm for 2 min. Bacterial pellets were re-suspended in 400 µl TE buffer (10 mMTris-HCl, 1 mM EDTA [pH 8.0]) followed by boiling for 10 min, cooling on ice for 10 min and centrifugation at 10,000 rpm for 10 min at 4 °C. Supernatant obtained after centrifugation was collected and used as the DNA template for PCR assay.

2.4. Molecular detection of E. coli

Polymerase chain reaction (PCR) was performed for the specific identification of *E. coli* targeting *malB* gene following the protocol described earlier (Wang et al., 1996). Briefly, a PCR reaction mixture was adjusted to 20 µl with 10 µl of 2X GoTaq® G2 Green Master Mix (Promega, USA), 10 pmol of each primer (Table S1, Supplementary file-2) and 2 µl of DNA templates. DNA extracted from *E. coli* strain ATCC25922 and *S. enteritidis* strain ATCC13076 was used as the positive and negative control, respectively. Amplification was conducted with an initial denaturation at 95 °C for 3 min followed by 30 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 60 s, and then a final extension step was conducted at 72 °C for 3 min on a thermal cycler (ASTEC 482, Japan).

2.5. Detection of major virulence determinants of diarrheagenic E. coli

E. coli isolated in this study were screened for the major virulence determinants viz. *stx*, *eae* and *cdt* genes of diarrheagenic *E. coli* using the primers enlisted (Table S1) using a multiplex PCR as described earlier (Hassan et al., 2019). The reaction mixture was adjusted to 20 µl as described earlier. PCR was performed on a thermal cycler (ASTEC, Japan) with initial denaturation at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s, and then a final extension step at 72 °C for 3 min.

2.6. Phylogenetic grouping of E. coli isolates

The phylogroup of *E. coli* strains was determined by triplex PCR (Clermont et al., 2000). Primers used in this study are enlisted in the Table (Table S1). PCR reaction was adjusted to 20 µl as stated

Table 1
Characteristics of the dairy farms included in this study.

Farms	No. of Dairy Cattle	Lactating cattle	Cattle with mastitis	Prevalence of mastitis
A	1535	450	14	3%
B	174	60	11	18.33%
C	225	75	16	21.33%
D	600	160	12	7.50%
Total	**2534**	**745**	**53**	**7.11%**

above. Amplification was carried out with an initial denaturation for 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, and a final extension step of 7 min at 72 °C on a thermal cycler (ASTEC, Japan). Phylogenetic group was defined as group A, B1, B2 and D according to the reference method.

2.7. Antimicrobial susceptibility testing

Antimicrobial susceptibility of the isolated *E. coli* was determined by disc diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI, 2018) and interpreted as susceptible, intermediate and resistant. A total of 14 antimicrobials comprising eight different antimicrobial classes commonly used in the dairy farms and human clinical cases in Bangladesh were selected in this study. Commercially available antibiotic disc (Oxoid, UK) namely aminoglycosides (Amikacin 30 µg – AK, Gentamicin 10 µg – GEN, Kanamycin 30 µg – K, Neomycin 30 µg – N), cephalosporins (ceftazidime 30 µg - CAZ, ceftriaxone 30 µg – CTR, cephalexin 30 µg – CN), fluoroquinolones (ciprofloxacin 5 µg – CIP), macrolides (Azithromycin 15 µg – AZM), penicillins (amoxicillin 10 µg – AMX, ampicillin 10 µg – AMP), phenicols (chloramphenicol 30 µg – C), polymyxins (colistin 10 µg – CL) and tetracyclines (tetracycline 30 µg – TE) were used in this study. The experiment was performed for at least three times to confirm the reproducibility of the results and *E. coli* strain ATCC25922 was used as the control strain in each experiment. Isolates showing resistance to three or more classes of antimicrobials are considered as multidrug resistant (MDR) (Magiorakos et al., 2012).

2.8. PCR detection of antimicrobial resistance genes

E. coli isolated in this study were screened for the presence of antimicrobial resistance genes by PCR. Based on the phenotypic resistance pattern, genes conferring resistance to β-lactams (bla_{TEM-1} and bla_{SHV-1}) and tetracyclines (*tetA*, *tetB* and *tetC*) were screened by PCR following the protocol described previously (Chen et al., 2004). PCR reaction mixture was adjusted to 20 µl as described earlier. The thermal profile included an initial denaturation at 95 °C for 10 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min and a final step consisting of 72 °C for 7 min.

2.9. Sequencing and analysis

16S rRNA gene of randomly selected *E. coli* isolates were amplified and sequenced using the primers 8F and 1492R (Table S1). Sequencing was performed using Sanger's sequencing technique on an Applied Biosystems 3500 series genetic analyzer (Thermo Fisher Scientific, USA). Acquired sequences were confirmed as *E. coli* by blast search (blast.ncbi.nlm.nih.gov/Blast.cgi).

2.10. Statistical analysis

2.10.1. Descriptive analysis

Data obtained from this study were incorporated into the Excel-2010 (Microsoft, Los Angeles, CA, USA) and exported to the Statistical Package for Social Science- SPSS (IBM SPSS 25, IBM, Chicago, IL, USA) for analysis. Fisher's exact test was used to determine the significant difference in the occurrence of *E. coli* in the milk samples collected from cattle with clinical mastitis and apparently healthy cattle. A p value < 0.05 ($p < 0.05$) was considered as the significant difference among the parameters. SPSS version 25.0 software (IBM Corp., Armonk, N.Y., USA) was used for the analyses.

2.10.2. Bivariate analysis

A Pearson correlation test was carried out with SPSS (version 25.0) to evaluate the associations in between any of two antibiotics which were resistant to *E. coli* isolates. A p-value less than 0.05 was deemed statistically significant.

3. Results

3.1. Isolation of E. coli in clinical mastitis of cattle

E. coli like colonies with characteristic metallic sheen on EMB agar were isolated from 19 (35.8%) out of 53 clinical mastitis samples (Table 2). Three colonies were selected from each sample and confirmed as *E. coli* by PCR targeting *E. coli* specific *malB* gene (Fig. S1, Supplementary file-1). The isolation was further confirmed by sequencing of 16S rRNA gene of randomly selected *E. coli* isolates (Accession numbers MW538946-MW538950). On the other hand, none of the 15 milk samples from apparently healthy cattle were positive for *E. coli* by cultural or molecular analysis (Table 2). The difference in the occurrence of *E. coli* in clinical mastitis and apparently healthy cattle milk was statistically significant ($p = 0.007$) (Table 2) indicating a possible association of the *E. coli* with the clinical mastitis in cattle.

3.2. Virulence determinants and phylogenetic group of E. coli

None of the nineteen (19) *E. coli* isolates were found positive for *stx*, *eae* or *cdt* genes. Simultaneously, PCR was performed targeting *chuA*, *yjaA* and DNA fragment TspE4.C2 for the phylogenetic grouping of the isolated *E. coli* (Fig. S2, Supplementary file-1). Out of 19 *E. coli* isolates examined Based on the PCR and interpretation using reference method, 12 isolates belonged to group A and 7 belonged to group B1 (Table 3).

3.3. Antimicrobial susceptibility

Antimicrobial susceptibility of 19 *E. coli* isolates (1 isolate from each positive sample) was determined against 14 antimicrobials of 8 different classes. Out of 19 isolates 16 (84.2%) were found multidrug resistant. Highest resistance was observed against amoxicillin (94.5%) followed by ampicillin (89.5%) and tetracycline (89.5%) (Fig. S3, Supplementary file-1). All the isolates were resistant to at least one of the β-lactam antibiotics (Table 3). Out of 16 multidrug resistant isolates 81.25 and 18.75% isolates were resistant to 3 and 4 classes of antimicrobials, respectively.

By bivariate analysis, positive significant correlations were identified in between resistance patterns against ampicillin and amoxicillin (Pearson correlation coefficient, $\rho = 0.687$; $p = 0.001$),

Table 2
Isolation of *E. coli* from Clinical mastitis of cattle.

Farms	Cattle with Clinical Mastitis		Apparently healthy cattle		P value
	No. of samples	*E. coli* positive (%)	No. of samples	*E. coli* Positive (%)	
A	14	7 (50.0)	4	0 (0)	0.119
B	11	3 (27.3)	3	0 (0)	1
C	16	5 (31.3)	5	0 (0)	0.278
D	12	4 (33.3)	3	0 (0)	0.516
Total	**53**	**19 (35.8)**	**15**	**0 (0)**	**0.007**

Table 3
Characteristics of the *E. coli* isolates recovered in this study.

Isolate ID	Virulence genes			Anbiotic resistance pattern	Antibiotic resistant genes					Phylogenetic group
	stx	*eae*	*cdt*		*tetA*	*tetB*	*tetC*	bla_{TEM-1}	bla_{SHV-1}	
BAU/MH/Bag-3101	–	–	–	AMP-AMX-TE	+	–	–	–	–	B1
BAU/MH/Bag-3108	–	–	–	AK-AMP-AMX-TE-GEN	+	–	–	–	–	A
BAU/MH/Bag-3109	–	–	–	AMP-AMX-GEN-K-TE	+	–	–	–	–	B1
BAU/MH/Bag-3110	–	–	–	AMP-AMX-K-TE	+	–	–	–	–	A
BAU/MH/Bag-3111	–	–	–	AK-AMP-AMX-GEN-K-N-TE	+	–	–	–	–	A
BAU/MH/Bag-3112	–	–	–	AMP-AMX-TE	+	–	–	–	–	A
BAU/MH/Bag-3113	–	–	–	AK-AMP-AMX-CIP-GEN-TE	+	–	–	–	–	A
BAU/MH/Bag-3127	–	–	–	AK-AMP-AMX-GEN-K-TE	+	–	–	+	–	A
BAU/MH/Bag-3128	–	–	–	AMP-AMX-N-TE	+	–	–	–	–	A
BAU/MH/Bag-3131	–	–	–	CAZ	–	–	–	–	–	B1
BAU/MH/Bag-3133	–	–	–	AMP-AMX-CN-TE	+	–	–	+	–	A
BAU/MH/Bag-3135	–	–	–	AMX-CAZ-N-TE	+	–	–	–	–	A
BAU/MH/Bag-3142	–	–	–	AMP-AMX-GEN-N-TE	+	–	–	+	–	A
BAU/MH/Bag-3149	–	–	–	AMP-AMX-AZM-CAZ-K-N	–	–	–	–	–	B1
BAU/MH/Bag-3153	–	–	–	AK-AMP-AMX-TE	+	–	–	+	–	B1
BAU/MH/Bag-3154	–	–	–	AK-AMP-AMX-GEN-TE	+	–	–	+	–	A
BAU/MH/Bag-3157	–	–	–	AMP-AMX-K-N-TE	+	–	–	+	–	B1
BAU/MH/Bag-3162	–	–	–	AMP-AMX-AZM-TE	+	–	–	–	–	A
BAU/MH/Bag-3163	–	–	–	AK-AMP-AMX-TE	+	–	–	+	–	B1

AK: Amikacin; Amp: Ampicillin, AMX: Amoxicillin; AZM: Azithromycin; CAZ: Ceftazidime; CIP: Ciprofloxacin; CN: Cephalexin; GEN: Gentamicin; K: Kanamycin; N: Neomycin; TE: Tetracycline.

amoxicillin and tetracycline (ρ = 0.687; p = 0.001), and gentamicin and amikacin (ρ = 0.548; p = 0.015). In addition, negative significant correlations were also identified in between resistance profiles of tetracycline and ceftazidime (ρ = −0.792; p < 0.001), ampicillin and ceftazidime (ρ = −0.792; p < 0.001), and amoxicillin and ceftazidime (ρ = −0.544; p = 0.016) (Supplementary Table S2).

At farm level, antimicrobial resistance was observed against at least 4 classes of antimicrobials. Resistance was observed against all the antimicrobial classes used at the farm level to treat disease conditions including mastitis (Table 4). Interestingly azithromycin resistance was observed in two farms where the drug was never been used for treating mastitis or any other diseases.

Table 4
Farm wise antimicrobial resistance pattern.

Farms	Antimicrobials used to manage mastitis and other disease conditions	Phenotypic resistance of *E. coli*	
		Antimicrobials	No. antimicrobial classes
A	GEN, N, PEN, STR, TE	AK, AMP, AMX, CIP, GEN, K, N, TE	4
B	AMX, CTX, GEN, N, PEN, TE	AK, AMP, AMX, CAZ, GEN, K, N, TE	4
C	CTX, GEN, PEN, STR, TE	AK, AMP, AMX, AZM, CAZ, CN, GEN, K, N, TE	5
D	CIP, GEN, N, TE	AK, AMP, AMX, AZM, GEN, K, N, TE	4

AK: Amikacin; Amp: Ampicillin, AMX: Amoxicillin; AZM: Azithromycin; CAZ: Ceftazidime; CIP: Ciprofloxacin; CN: Cephalexin; CTX: Ceftriaxone; GEN: Gentamicin; K: Kanamycin; N: Neomycin; STR: Streptomycin, TE: Tetracycline.

3.4. Detection of antimicrobial resistance genes

In PCR all the tetracycline resistant *E. coli* (17/17) were found to carry *tetA* gene (Fig. S4, Supplementary file-1), but no *tetB* or *tetC* (Table 3). On the other hand, bla_{TEM-1} was detected in 38.88% (7/18) of the *E. coli* isolates (Fig. S5, Supplementary file-1, Table 3). None of the isolates were positive for bla_{SHV-1} gene (Table 3).

4. Discussion

E. coli is one of the major etiologies of bovine clinical mastitis having increased prevalence in the recent years (Green et al., 2005). *E. coli* infection in the mammary gland is considered as temporary and self-limiting; however, recurrence and persistent infection also have been reported (Döpfer et al., 1999; Hill et al., 1978; Hill and Shears, 1979; Hogan et al., 1989; Lam et al., 1996; Lipman et al., 1995). Recurrence of *E. coli* infection is thought to occur via reinfection from the nature or as a result of persistence of the organism within the mammary gland (Bradley and Green, 2001). Results showed that recurrence due to persistence of the *E. coli* in the mammary gland is more likely than recurrence from the nature (Bradley and Green, 2001). Recurrence or persistence of *E. coli* infection might depend on its ability to adhere to, and invasion to mammary epithelium (Dogan et al., 2006; Döpfer et al., 2000). In addition, several intestinal and extra intestinal virulence factors (*stx*, *eaeA*, *astA*, *cnf*, *papC*, *iucD*, *hlyA*, *ehx* etc.) have been detected in *E. coli* isolated from bovine mastitis which might contribute to

the pathogenesis of *E. coli* mastitis, however, their association with the severity and persistence is not yet clearly understood (Blum et al., 2015; Fernandes et al., 2011; Guerra et al., 2019). Antimicrobial resistance is another potential factor which might play critical role in the persistence of *E. coli* in the udder environment and result in the failure of antimicrobial therapy. Thus, virulence profile and antimicrobial resistance is critical to understand the pathogenesis of *E. coli* in clinical mastitis. Although several research have been performed throughout the world, none of the studies in Bangladesh have reported the virulence profile, phylogroup and antimicrobial resistance of *E. coli* isolated from clinical mastitis. Thus, the present study was prompted to determine the virulence determinants, phylogroup and antimicrobial resistance pattern of *E. coli* isolated from clinical mastitis in cattle.

E. coli was recovered from 35.8% of the mastitis samples examined in this study but none of the milk samples from apparently healthy cattle indicating that the *E. coli* might be associated with the mastitis in the cows included in this study. The occurrence recorded in this study was higher than that reported earlier in different parts of the world (Lan et al., 2020; Rahman et al., 2013; Zhang et al., 2018), however, as the farm selected in this study had persistent mastitis problem, higher occurrence of mastitis is not surprising. In addition, sample sizes and geographical locations might have influenced the findings.

Virulence of *E. coli* is associated with the pathogenesis in bovine clinical mastitis. In addition to the virulence determinants phylogenetic grouping of *E. coli* is critical to understand the emergence of new subgroups of virulent bacteria (Picard et al., 1999). In this study, *E. coli* isolates were screened for the major virulence determinants (*stx*, *eae* and *cdt* genes) of diarrheagenic *E. coli*. None of the *E. coli* isolates were positive for *stx, eae or cdt* genes. Absence of *stx* genes in the *E. coli* is in agreement with the findings reported earlier China, Iran and Belgium (Lan et al., 2020; Mansouri-Najand and Khalili, 2007; Vivegnis et al., 1999). However, occurrence of *stx* genes in *E. coli* have also been reported from bovine clinical and subclinical mastitis (Claeys et al., 2013; Jayarao et al., 2006; Little et al., 2008; Momtaz et al., 2012; Ombarak et al., 2019; Pradel et al., 2008; Van Kessel et al., 2011). In addition to *stx*, occurrence of *eae* in *E. coli* isolated from bovine mastitis milk was also reported by several authors (Lan et al., 2020; Momtaz et al., 2012). Thus, further studies with a greater number of samples are necessary to ascertain the presence of *stx*, *eae* and *cdt* gene in *E. coli* isolated from bovine clinical mastitis in Bangladesh.

The *E. coli* isolated in this study belongs to phylogenetic group A (63.2%) and B1 (36.8%) which is in consent with the findings of Tomazi et al. (2018), who have reported the occurrence of group A and B1 *E. coli* as 52 and 38%, respectively in bovine clinical mastitis. Group A an B1 *E. coli* belong to commensal *E. coli.* To the best of our knowledge this is the first study in Bangladesh describing the phylogenetic grouping of *E. coil* from mastitis. Findings of this present study show that mastitis causing *E. coli* detected in this study are typical commensals (Suojala et al., 2011). Moreover, this finding is also evidence on involvement of commensal *E. coli* as the etiology of bovine clinical mastitis.

Antimicrobial resistance is critical to understand the pathogenesis and selection of proper antimicrobials to mitigate *E. coli* mediated mastitis (Blum et al., 2008). About 84.2% (16/19) *E. coli* isolated in this study were multidrug resistant with highest resistant of amoxycillin followed by ampicillin and tetracycline which coincides with the findings described earlier (Lan et al., 2020). However, the level of resistance to ampicillin and tetracycline observed in this study was higher than those reported previously (Lan et al., 2020; Tark et al., 2017; Zhang et al., 2018). All the *E. coli* isolates of this study were resistant to at least one antimicrobial class used in the study farms. Furthermore, positive significant correlations in between resistance profiles of ampicillin and amoxicillin, amoxicillin and tetracycline, and gentamicin and amikacin; and negative significant correlation in between resistance patterns of tetracycline and ceftazidime, ampicillin and ceftazidime, and amoxicillin and ceftazidime were observed by bivariate analysis. Antimicrobial resistance pattern of the *E. coli* isolates correlates with the antimicrobial used in the respective farms indicating an overuse or misuse of antimicrobials might be associated with the development of resistance. However, phenotypic azithromycin resistance was observed in two *E. coli* isolated from two different farms where there was no history of use of azithromycin for treatment of mastitis or other health conditions. This finding suggests that misuse of antimicrobials probably not only the single factor involved in the antimicrobial resistance development in the *E. coli* strains (Bergman et al., 2009; Oliver et al., 2011).

The antimicrobial resistance genotypes against amoxicillin/ampicillin and tetracycline was determined by PCR. About 38.9% of the amoxicillin and/ or ampicillin resistant isolates carried bla_{TEM-1} but none of them were positive for bla_{SHV-1} which supports the findings of Tahar et al., (2020) who also reported increased prevalence (30.7%) of bla_{TEM-1} in *E. coli* isolated from bovine clinical mastitis in Algeria. This study also indicates that not only bla_{TEM-1} other β-lactam resistant genotypes might be present in the *E. coli* isolates. Thus, a detailed investigation comprising all the β-lactam genes described so far is crucial to determine the overall β-lactam resistance genotypes circulating in the *E. coli* isolated from clinical mastitis in Bangladesh. Findings of such study will help in selecting effective antibiotics for better mastitis treatment. On the other hand, phenotypic resistance to tetracycline was 100% correlated to its genotypic resistance. All the tetracycline resistant *E. coli* carried *tetA* gene, however, no *tetB* or *tetC*. Our study supported the findings who have reported increased prevalence of *tetA* than *tetB* or *tetC* genes (Gomi et al., 2017; Jianying et al., 2008; Lan et al., 2020; Rebbah et al., 2018).

5. Conclusion

This study demonstrated that *E. coli* isolated from clinical bovine mastitis are typical commensal. They did not carry major virulence determinants (*stx*, *eae* and *cdt* genes) of diarrheagenic *E. coli*. Almost all the isolates are multidrug resistant which might be associated with the overuse of respective antibiotics to control mastitis or other disease condition of the affected animals. Occurrence of multidrug resistant *E. coli* is alarming and indicates a potential risk of transferring multidrug resistant *E. coli* and resistance to human, animal and nature through the contamination milk or milk products. However, due to limitation in the sampling procedure, the number of farms and geographical areas selected, the actual scenario of *E. coli* genotypes and antimicrobial resistance phenomenon prevailing in Bangladesh could not be ascertained. Thus, further in depth phenotypic and genotypic analysis with a greater number of samples and *E. coli* isolates are suggested.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.06.099.

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