JABET
Journal of Advanced Biotechnology and Experimental Therapeutics

ORIGINAL ARTICLE

J Adv Biotechnol Exp Ther. 2024 May; 7(2): 336-345
eISSN: 2616-4760, https://doi.org/10.5455/jabet.2024.d28
Published by www.bsmiab.org

Characterization of *tet*A gene of *Escherichia coli* isolated from colibacillosis affected calves in Rangpur, Bangladesh

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Academic editor
Md. Masudur Rahman, PhD
Obihiro University
Japan

Article info
Received: 07 January 2024
Accepted: 20 February 2024
Published: 02 March 2024

Keywords
Antibiotic resistance, Colibacillosis, *E. coli*, *tet*A gene, Tetracycline

ABSTRACT

Antimicrobial resistance is a matter of threat in global public health nowadays as a great number of antibiotics have lost their potential. The current study aimed to characterize the *tet*A gene involved in tetracycline resistance of *Escherichia coli* in colibacillosis affected calves. To perform the study, a total of 69 fecal samples from colibacillosis suspected diarrheic calves were collected randomly from different areas of Rangpur division in Bangladesh. Standard laboratory protocols were followed for isolation and identification of *E. coli*. Then bacterial sensitivity was tested by culturing the bacteria in Mueller Hinton agar by following the protocol of Kirby-bauer disc diffusion method. Tetracycline resistant gene was specified by using *tet*A primer by searching *tet*A gene and the resistant gene was sequenced by sanger sequencing method. A phylogenetic tree of nucleotide sequences of *tet*A gene of *E. coli* was formulated using NCBI blast to characterize the gene. From 69 fecal samples, 54 were positive for *E. coli*. In antibiogram study, 100% isolates of *E. coli* were resistant to amoxicillin, ampicillin, cefoxitin, erythromycin and imipenem and the nature of the isolates of *E.coli* against other antibiotics were as follows: doxycycline 64.8%, co-trimethoprim 63%, tetracycline 59.3%, ceftriaxone 50%, gentamicin 16.7%, levofloxacin 14.8% respectively. This study noticed that about 56.25% samples (18 from 32) were resistant to tetracycline due to *tet*A gene and the gene (*tetA* from *E. coli*) was closely related to the corresponding gene of *Klebsiella pneumoniae*, *Shigella sonnei*, *Proteus mirabilis* and *Citrobacter braakii* strains besides *E. coli*. This study suggests determining the other genes that are specific for tetracycline resistance in *E. coli* in Rangpur for better understanding the nature of it.

INTRODUCTION

Calves are affected by a large number of infectious and non-infectious diseases. Among these, *Escherichia coli* causing colibacillosis is regarded as one of the most important problems in the dairy industry which has a negative impact on a nation's economy [1]. From environmental sources, *E. coli* colonizes in the mammalian intestinal tract just after birth and these organisms remain as important members of the normal intestinal flora throughout life [2]. *E. coli* affects calves below 2 months old and the bacteria are prevalent in farms according to the farm location, calf management, and herd size [3, 4]. Colibacillosis-affected calves normally show diarrhea, dehydration, metabolic acidosis and fever with meningitis and arthritis can occur as a sequel in some cases. However, typical signs noticed in colibacillosis are fever, diarrhea, and dehydration [5].

Although *E. coli* is normally present in the intestine as an essential flora, a variety of enteric infections (colibacillosis is one of them) can occur which can lead to life-threatening conditions hence antimicrobial therapy is needed [6]. But most of the time, the farm owners use antibiotics without consulting any registered veterinarians, and the animals are not tested whether they have relevant disease or not. Moreover, the overuse of antibiotics in animals is a very important cause of antimicrobial resistance. The indiscriminate use of antimicrobial agents by farm owners is a common malpractice triggering the progression of multi-drug resistant bacteria [7].

Several studies suggest that penicillin, tetracyclines, sulfa drugs, and cephalosporins are at risk for antimicrobial resistance [8, 9]. Antibiotic resistance is interlinked between humans and farm animals because resistant organisms can easily be transmitted to humans in different ways. Therefore, antimicrobial resistance in animals is one of the biggest issues of the 21st century [10]. Every year, near about 0.7 million humans are gone towards death throughout the world of it. This number can be enhanced in the future if effective antibiotics are not discovered [11]. So, it creates attention for healthcare personnel as well as economic experts to prevent the acquired antibiotic resistance [12].

Specific genes of micro-organisms are responsible for each of the antibiotic resistance. *E. coli* has numerous genes that are responsible for resistance to beta-lactam antibiotics. Narrow-spectrum β-lactamases coded by *blaTEM-1* can inactivate penicillin and aminopenicillins and this gene is widespread in *E. coli* from animals [13]. Tetracycline resistance is predominant in *E. coli* due to tet genes including *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, and *tetG* where 80% of *E. coli* isolates are resistant due to *tetB* gene [14]. Although the amplitude of tetracycline-resistant genes between *tetA* and *tetB* is fluctuating from one form to another, *tetA* gene is predominant than *tetB* gene in Bangladesh [15, 16]. Previously, very limited study focused on determining resistant genes responsible for tetracycline resistance in Bangladesh. This study aimed to characterize tetracycline resistant gene of *E. coli* due to *tetA* gene. The rest of the genes which are engaged in tetracycline resistance should be characterized in future to fulfill the intention of the present study.

MATERIALS AND METHODS

Ethical approval

The ethical approval was provided by Hajee Mohammad Danesh Science and Technology University (HSTU/IRT/4215).

Collection and processing of samples

From January 2022 to December 2022, 40 farms in the selected areas were continuously supervised to collect 69 fecal samples from colibacillosis suspected calves with fever, diarrhea, and general weakness in different parts of Rangpur, Bangladesh. Among those samples, 41 were collected from males, and 28 were collected from females. In terms of age, the sample size of <1 week, 1-3 weeks and 3-6 weeks were 21, 33, and 15 respectively. The samples were taken immediately after defecation or directly from the rectum by using sterile gloves. Then the samples were taken in a dry and clean special vial containing phosphate buffered solution (HI MEDIA, Mumbai, India) with tagging relevant information. The samples were fresh and transferred immediately from the farms to the laboratory in an ice box containing ice gel [17, 18]. In the laboratory, fecal samples were stored at 4°C until the preparation of culture media. All the samples were inoculated within the clean bench by maintaining an aseptic procedure. Sample collection to inoculation was done within 24 h [19].

Bacterial culture and biochemical tests

By using a sterile platinum loop, the collected fecal samples were directly inoculated by streaking onto nutrient agar media (HI MEDIA, Mumbai, India) and incubated at 37°C for 24 h. Later some colonies from nutrient agar media were streaked onto MacConkey

agar (HI MEDIA, Mumbai, India) and then Eosin Methylene Blue (EMB) agar media (HI MEDIA, Mumbai, India) while incubating at 37°C for 24 h. The colonies from EMB agar were sub-cultured in the same media several times to produce a pure culture of *E. coli* [20]. After that, the bacterium was identified by performing gram's staining technique and several biochemical tests like as catalase, oxidase, methyl red, Voges Proskauer (VP), indole, citrate utilization, triple sugar iron (TSI), motility-indole-urease (MIU) according to the former study [21]. Here all the reagents used in the biochemical test were collected from HI MEDIA, Mumbai, India.

Amplification of *16s* and *23s rRNA* gene of *E. coli*

For molecular confirmation, the *16s and 23s rRNA* genes of *E. coli* were targeted by Eco-223 and Eco-455 primers designed through polymerase chain reaction (PCR) in a thermocycler (Biotech, India) as shown in Tables 1 and 2. To do this, a heating method was followed for extracting desired DNA from *E. coli* [22]. Previous protocols were followed for setting PCR conditions and gel documentation where GoTaq® Green master mix, 2x (Promega, USA) was used [23, 24].

Antibiotic-resistant profiling

Antibiotic sensitivity test was performed according to Kirby-Bauer disc diffusion method [25]. Pure culture from EMB agar plates was cultured onto Mueller-Hinton agar (HI MEDIA, Mumbai, India) by streaking with different antibiotic discs (amoxicillin, ampicillin, ceftriaxone, cefoxitin, streptomycin, gentamicin, erythromycin, tetracycline, doxycycline, chloramphenicol, ciprofloxacin, enrofloxacin, levofloxacin, imipenem, and co-trimethoprim) and incubated at 37°C for 24 h. All the antibiotic discs were imported from HI MEDIA, Mumbai, India. The zone of inhibition was calculated to millimeter unit and compared with clinical and laboratory standard institute (CLSI), 2023 to make antibiotic-resistant profile of *E. coli* [26].

PCR amplification of *tetA* gene

DNA extracts from 32 tetracycline-resistant isolates of *E. coli* were taken for *tetA* gene identification. *tetA* gene responsible for tetracycline resistance was identified by using tetA primer through PCR amplification. PCR reaction for detection of *tetA* gene was performed by setting the standard PCR condition with the following steps: initial denaturation for a single cycle with repeated 30 cycles each having denaturation, annealing, and final extension for a single cycle (Table 1 and 2). That was performed according to the method described formerly by several authors [27, 28]. The amplified PCR product was run through 1.5% agarose gel containing ethidium bromide at 85 volts for 45 minutes. After the dye line ran approximately 75-80% of the gel, DNA fragments were visualized by using a gel documentation machine (UVITEC, United Kingdom) which emits UV light.

Table 1. Primers used in the present study.

Primer name	Gene identified	Primer sequence	Band size	References
Eco-223 and Eco-455	*16s rRNA*	F: ATCAACCGAGATTCCCCCAGT R: TCACTATCGGTCAGTCAGGAG	232 bp	[22]
tetA	*tetA*	F: GGTTCACTCGAACGACGTCA R: CTGTCCGACAAGTTGCATGA	577 bp	[26, 27]

F: Forward primer, R: reverse primer

Table 2. Conditions of PCR for Eco-223, Eco-455 and tetA primers.

Steps	Temperature and duration for Eco-223 and Eco-455 primer	Temperature and duration for tetA primer	Cycle
Initial denaturation	95°C for 5 minutes	95°C for 5 minutes	1 cycle
Denaturation	95°C for 1 minute	94°C for 1 minute	
Annealing	56°C for 40 seconds	57°C for 30 seconds	30 cycles
Extension	72°C for 1 minute	72°C for 1 minute	
Final extension	72°C for 5 minutes	72°C for 5 minutes	1 cycle
Holding	4°C until use	4°C until use	-

Single cycle for Initial denaturation; 30 cycles for Denaturation, Annealing, Extension; Single cycle for Final extension

Sequencing *tetA* gene and analysis of the phylogenetic tree

According to the previous study, the *tetA* gene was sequenced (2 PCR products were used) by Sanger sequencing method by using tetA forward primer [29]. The sequenced data were edited, and gene fragments were assembled in the BioEdit software, and the data were submitted to the gene bank. The accession number provided by the gene bank was OR450016. The *tetA* sequences were performed blast on the NCBI website. Additional *tetA* gene sequences were retrieved from the Genbank and included in the data set. Multiple sequence alignments were done using ClustalW performed in the BioEdit software package. MEGA11 software was used for the construction of a phylogenetic tree and multiple sequence alignment was performed to align the sequences using the MUSCLE algorithm. The Neighbor-Joining root method was used with the maximum composite likelihood nucleotide substitution mode. 500 Bootstrap validity score was considered for the construction of the tree.

RESULTS

Properties of *E. coli* in cultural and biochemical studies

After incubation at 37°C for 24 h, a rose-pink colony appeared in MacConkey agar media, and in EMB agar plates, the colony showed a characteristic green metallic sheen (Figure 1). After several sub-cultures, the organism was found as a small rod under a microscope using the Gram staining technique. In biochemical tests, the isolates were positive for catalase, methyl red, indole, motility test, and gas production and negative for VP, citrate utilization, and urease test (Figure 2). 232 bp band was noticed in UV exposure after PCR amplification that ensured the presence of *E. coli* (Figure 3). Out of 69 fecal samples, 54 were positive for *E. coli* based on cultural, biochemical, and PCR results.

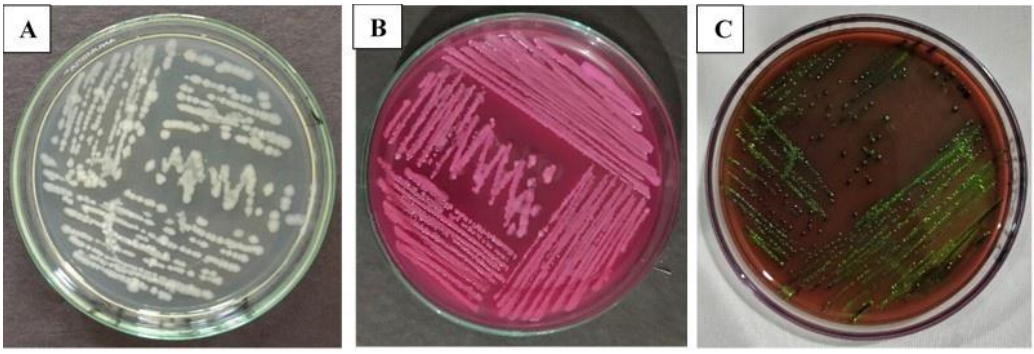

Figure 1. Cultural characteristics of the isolates of *E. coli* are as follows: grayish white, circular, opaque colonies were appeared in nutrient agar media (A), rose pink colored colonies in MacConkey agar media (B) and characteristic green metallic sheen-colored colonies were observed in Eosin methylene blue agar media (C).

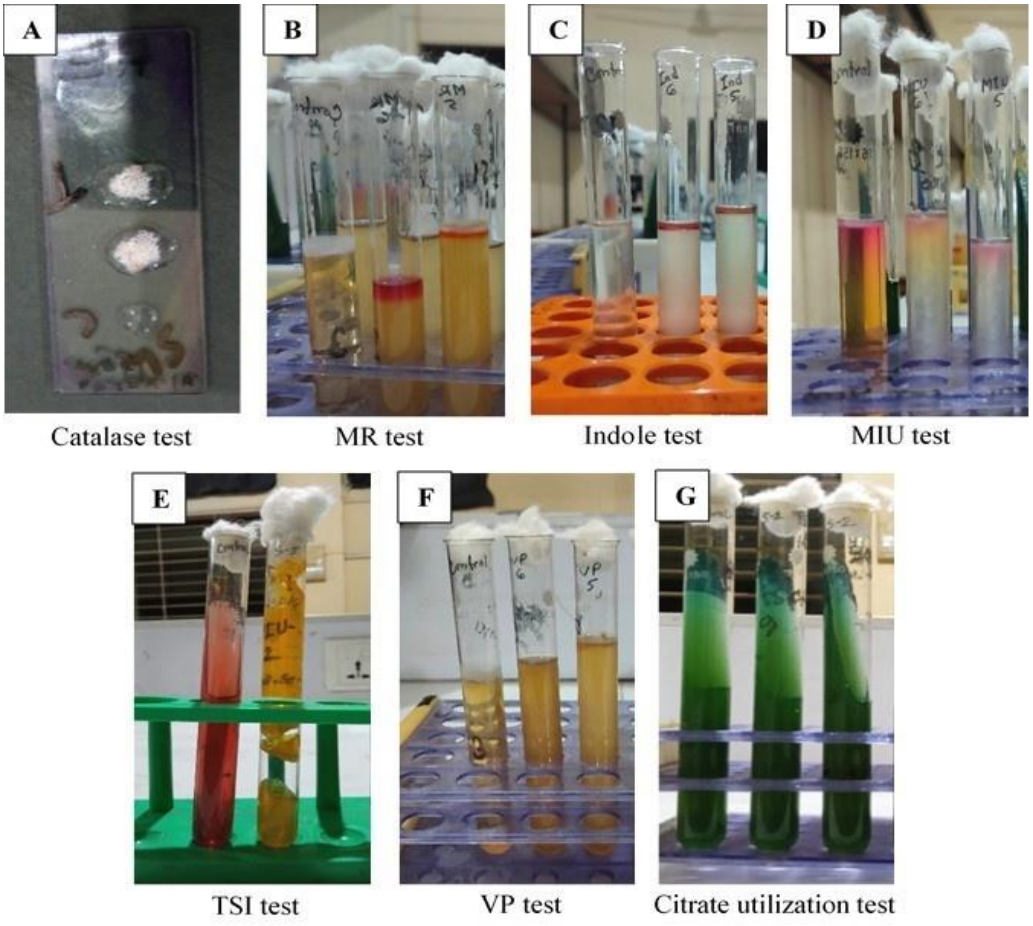

Figure 2. After observing the cultural characteristics of *E. coli*, several biochemical tests were performed. The properties of *E. coli* seen in different biochemical tests are as follows: bubbles were formed by *E. coli* indicating oxidase positive (A), red colored ring was visualized in methyl red test (B), pink colored ring in Indole test (C), the isolates were motile and urease was negative in MIU media (D), slant and butt were acidic and huge amount of gas was formed in TSI media (E), no changes were observed in VP media (F) and in citrate utilization test, *E. coli* was also negative (G).

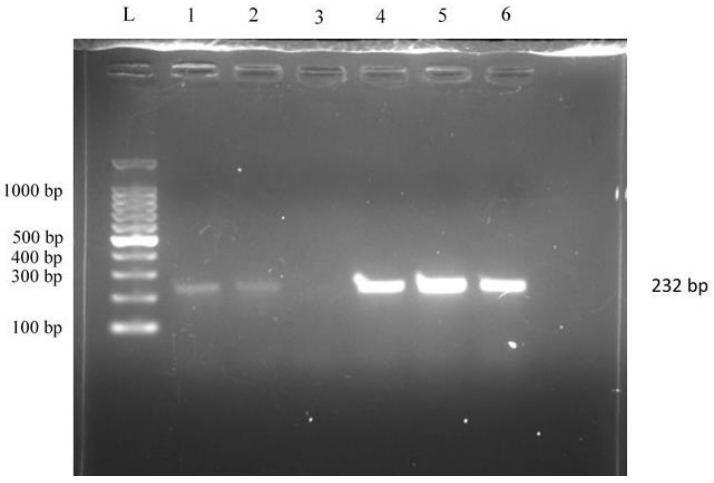

Figure 3. Confirmation of *E. coli* by observing 232bp band through amplification of *16s rRNA* gene by PCR. Lane L: 1kb DNA ladder; Lane 1-6: DNA samples of bacteria isolated from different calves.

Antibiotic resistance profile of *E. coli*

54 samples were tested by the disc diffusion method for antibiotic sensitivity. 100% of the isolates of *E. coli* were resistant to amoxicillin, ampicillin, cefoxitin, erythromycin, and imipenem, and the resistant pattern against other antibiotics were as follows: doxycycline (64.8%), co-trimethoprim (63%), tetracycline (59.3%), ceftriaxone (50%), gentamicin (16.7%), chloramphenicol (16.7%), and levofloxacin (14.8%). The overall results of antibiotic sensitivity are shown in Table 3.

Table 3. Antibiotic resistant profile of *E. coli.*

Antibiotic class	Antibiotic name	Disc name with conc.	Total number of isolates (%)		
			Sensitive	Intermediate	Resistant
Penicillin	Amoxicillin	AMX-30	0(0%)	0(0%)	54(100%)
	Ampicillin	AMP-25	0(0%)	0(0%)	54(100%)
Cephalosporin	Ceftriaxone	CTR-30	23(42.6%)	4(7.41%)	27(50%)
	Cefoxitin	CX-30	0(0%)	0(0%)	54(100%)
Aminoglycosides	Gentamicin	GEN-10	18(33.3%)	27(50%)	9(16.7%)
	Streptomycin	S-10	28(51.8%)	26(48.2%)	0(0%)
Macrolides	Erythromycin	E-15	0(0%)	0(0%)	54(100%)
Tetracycline	Tetracycline	TE-30	0(0%)	22(40.7%)	32(59.3%)
	Doxycycline	DO-30	10(18.5%)	9(16.7%)	35(64.8%)
Phenicol	Chloramphenicol	C-30	45(83.3%)	9(16.7%)	0(0%)
Fluoroquinolones	Ciprofloxacin	CIP-5	33(61.1%)	21(38.9%)	0(0%)
	Enrofloxacin	EX-5	43(79.6%)	11(20.4%)	0(0%)
	Levofloxacin	LE-5	31(57.4%)	15(27.8%)	8(14.8%)
Carbapenems	Imipenem	IMP-10	0(0%)	0(0%)	54(100%)
Sulfa-drug	Co-trimethoprim	COT-25	14(26%)	6(11%)	34(63%)

The total number of samples was 54.

Prevalence of *tetA* gene

PCR amplification was done for the purpose of identifying the *tetA* gene responsible for tetracycline resistance. 32 samples (the isolates that were visually resistant against tetracycline in the disc diffusion method) were run under PCR, in which 18 samples were positive (577 bp band was visualized) for the *tetA* gene, indicating that 56.25% of isolates of *E. coli* were resistant against tetracycline due to the *tetA* gene (Figure 4). Of 18 positive isolates, 5 were collected from the <1-week group, 9 were collected from the 1-3-week group, and 4 were collected from the 3-6-week group.

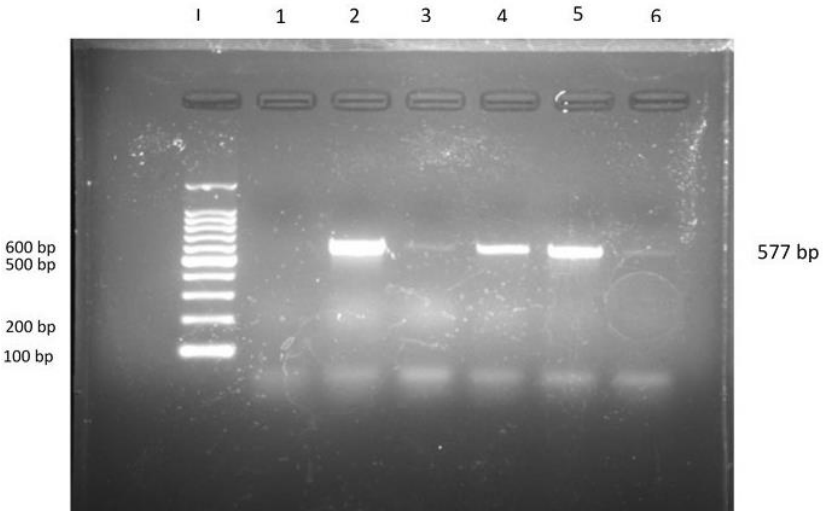

Figure 4. Identification of tetracycline resistant gene of *E. coli* by amplification of *tet*A gene through PCR indicating 577bp. Lane L: 1kb DNA ladder; Lane 1-6: DNA of bacteria isolated from different calves.

Clade of *tetA* gene in phylogenetic tree analysis

To further investigate the genetic relationship of the *E. coli tetA* gene, a phylogenetic tree was constructed. According to Figure 5, the gene sequence was homologous to *E. coli* strain GN03204 plasmid p3204 as well as the strains formerly observed at HSTU, including *E. coli* strain G1097 plasmid, *E. coli* strain XYEH2934 plasmid, and *E. coli* strain MS1050 plasmid. Besides *E. coli,* the gene sequence was also closed to the genes of *Shigella sonnei* strain 202008564 plasmid, *Proteas mirabilis* strain SDM004 plasmid pSDM004, *Klebsiella pneumoniae* strain TSNTC10-1 plasmid, and *Citrobacter braakii* strain LBA3.

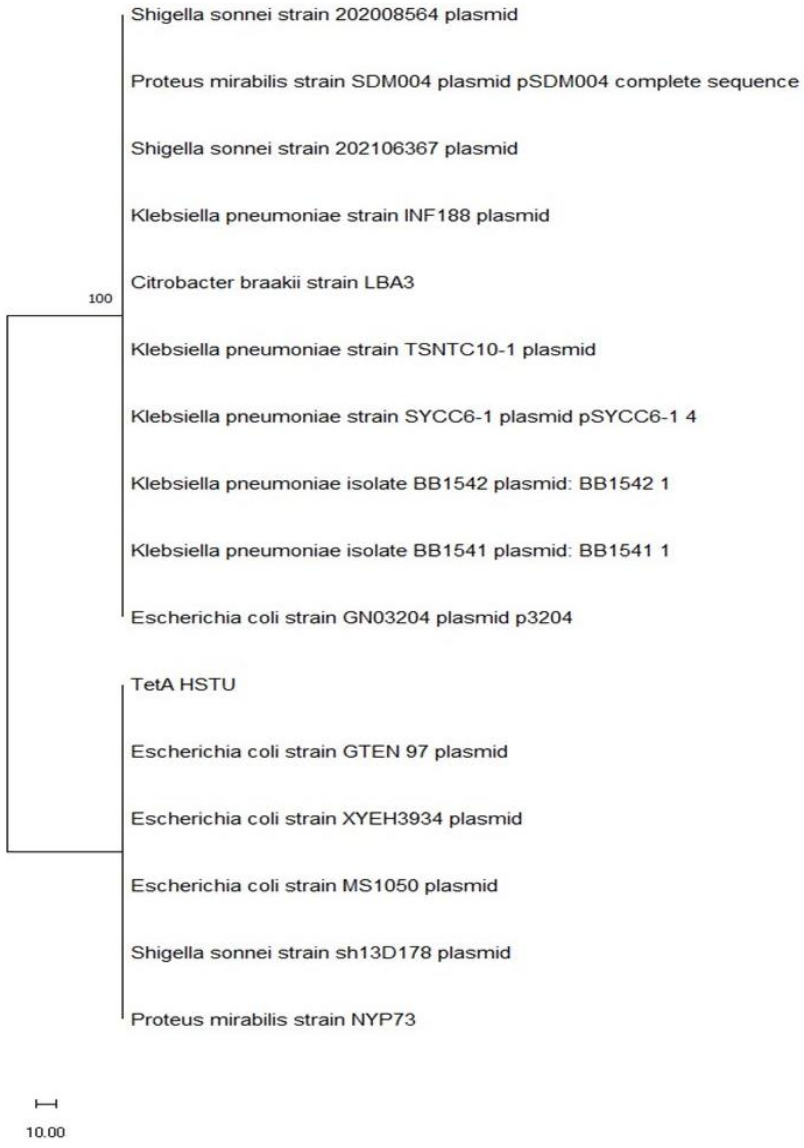

Figure 5. Phylogenetic analysis of *tet*A gene based on the nucleotide sequences. The Neighbor-Joining method with maximum composite likelihood nucleotide substitution model was followed by using MEGA11 software. The isolates sequence in this study is *tet*A HSTU which have the same clade as the previous studies.

DISCUSSION

Calf diarrhea, mainly calf colibacillosis, in Rangpur is a major problem in the dairy industry like the other division of Bangladesh. In most cases, the farm owner tends to use antibiotics without maintaining proper dosage [30]. As a result, antibiotic resistance is a common issue in Rangpur nowadays. Antibiotics are mainly used to treat bacterial

infections in humans and animals as they have the ability to kill the bacteria or suppress their growth. Penicillin was discovered almost 100 years ago but in the present time, physicians feel they lack the weapons to fight infections [31]. This study aimed to study the antibiogram pattern of *E. coli* causing colibacillosis by determining the role of the *tetA* gene in the resistance of *E. coli* against tetracycline.

To observe the matter, diarrheal samples from colibacillosis-affected calves were tested for *E. coli* existence. The samples were cultured on different media (Nutrient agar, MacConkey agar, and EMB agar) to obtain a pure culture of *E. coli*. A rose pink and green metallic sheen colored colony was found in MacConkey and EMB agar, which was supported by the former studies [32, 33]. Some colonies were taken from EMB agar with a sterile platinum loop and made a thin smear on glass slide to perform gram's staining technique where the organism was visible as pink colored rod indicating gram negative organism most probably *E. coli* and that was identical to previous study [34]. From EMB and nutrient broth, different biochemical tests were performed to confirm the accuracy of *E. coli*. The bacterial cultures were positive to catalase, methyl red, indole, motility test & gas production and were negative to VP, citrate utilization, urease as well as oxidase test supported by the former researcher [35].

After *E. coli* confirmation, an antibiotic-resistant profile was made. In this study, 100% of the isolates of *E. coli* are resistant to amoxicillin, ampicillin, cefoxitin, erythromycin, and imipenem, which indicates a mammoth threat to the health sector in the near future. This study also tried to examine *E. coli* against some other important antibiotics, and the results are as follows: doxycycline (64.80%), co-trimethoprim (63.00%), tetracycline (59.30%), ceftriaxone (50.00%), gentamicin (16.70%), and levofloxacin (14.8%). Previously, several studies had been done where they found the same in the cases of amoxicillin and ampicillin, but they found *E. coli* 43.18% resistant to erythromycin. On the contrary, the isolates of *E. coli* were 100% resistant to ampicillin, tetracycline, co-trimoxazole, and erythromycin [36, 37].

Previously, many scientists tried to find out the prevalence of different genes associated with tetracycline resistance in *E. coli*. In 2005–06, researchers found 54.4% of isolates of *E. coli* were resistant to tetracycline, 8.1% of which occurred due to the *tetA* gene and 86.5% due to the *tetB* gene. In 2010–11, *E. coli* resistance was 47.1% to tetracycline, and at that time, a clear fluctuation between the *tetA* and *tetB* genes was noticed. *tetA* was more dominant than tetB, and the percentage was 81.3% for *tetA* and 18.7% for the *tetB* gene [16]. The present study notices that 59.3% of isolates of *E. coli* are resistant against tetracycline, and this is higher than the previous record. The prevalence of the *tetA* gene is 56.25%, which is intermediate between the results of the two studies stated before. The same study was performed in 2019, where tetracycline was 100% resistant to *E. coli* and 77.80% of isolates were positive for the *tetA* gene [38]. In phylogenetic analysis, the *tetA* gene of *E. coli* isolated from colibacillosis-affected calves in Rangpur, Bangladesh, found the same clade as other *E. coli* plasmids from the animals.

As a multiple number of genes are responsible for the resistance of *E. coli* to tetracycline, demarking a single gene (*tetA*) cannot express the full idea of its nature. So, it should be emphasized to detect the other genes to accomplish the study goal.

CONCLUSION

From the present findings, the isolates of *E. coli* were resistant to multiple antibiotics; *E. coli* was found to be 100% resistant to amoxicillin, ampicillin, cefoxitin, erythromycin, and imipenem, indicating an alarming threat in the near future. 59.3% of *E. coli* isolates

were found resistant to tetracycline in the disc diffusion method, while 56.25% of isolates (18 out of 32) were resistant due to the *tetA* gene. After confirmation of the *tetA* gene by PCR amplification, the *tetA* gene responsible for tetracycline resistance was sequenced, and this concludes that the sequenced gene was nearly similar to the gene present in the strains *Klebsiella pneumoniae, Shigella sonnei, Proteus mirabilis,* and *Citrobacter braakii,* besides *E. coli*, hence warning that a similar mechanism was present among them for tetracycline resistance. This study suggests antibiotics should be used only after sensitivity testing with proper dosage in order to avoid antibiotic resistance.

ACKNOWLEDGEMENT

The author would like to acknowledge the Ministry of Science and Technology, Government of the People's Republic of Bangladesh, for their tangible support as they selected the author for an NST scholarship to conduct the research work fruitfully (NST 2021-22/R225/M314). The author is also thankful to his research supervisor, Md Mustafizer Rahman, and co-supervisor, Nazmi Ara Rumi, for their expertise, active supervision, inspiration, and valuable suggestions to complete the research work.

AUTHOR CONTRIBUTIONS

MMR and NAR provided the study design and concept; JHJ and NAR performed the laboratory work (both gross and molecular); JJH and NAR wrote down the main manuscript; and MMR reviewed the manuscript. All authors approved the final version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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