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Prevalence of multidrug-resistant ESBL-producing *Escherichia coli* isolated from beef and sheep meat in Sylhet, Bangladesh

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ABSTRACT

Nowadays, the spread of antibiotic-resistant *Escherichia coli (E. coli)* from food animals to humans is considered to be a serious public health problem. The aim of the study was to determine the prevalence of multidrug-resistant ESBL-producing *E. coli* isolated from beef and sheep meat. A total of 400 meat samples (200 beef and 200 sheep) were randomly collected from different slaughterhouses and wet markets in Habiganj, Sylhet, Moulvibazar, and Sunamganj districts of the Sylhet division of Bangladesh. Among 400 samples, 136 *E. coli* were isolated from meat samples (90 beef and 46 sheep). Disc diffusion antimicrobial susceptibility assay was used to test the antimicrobial susceptibility traits of *E. coli*. The overall prevalence of multidrug resistance was 56.67% in *E. coli* of beef samples and 43.47% in *E. coli* of sheep meat. *E. coli* isolates of the meat samples (beef and sheep meat) were found to be 100% resistant to both Erythromycin and Ampicillin (100%), and 100% sensitive to Cotrimoxazole, Ciprofloxacin, Gentamycin, Levofloxacin, and Colistin. Furthermore, antibiotic sensitivity tests were performed using Cefotaxime, Ceftazidine, Ceftriaxone, and Aztreonam to know the prevalence of ESBL producers in isolated *E. coli.* ESBL-producing *E. coli*, which showed resistance to both Cefotaxime and Ceftriaxone, was found at 21.11 % (19/90) and 4.35% (2/46) in beef and sheep meat respectively. Our results showed that the best drugs to treat animals afflicted with ESBL-producing *E. coli* were Ceftazidine and Aztreonam, highlighting the urgent need to minimize the unnecessary use of antibiotics.

INTRODUCTION

According to the World Health Organization (WHO), antimicrobial resistance (AMR) has become a significant worldwide health issue confronting humanity [1]. Current estimates place the number of AMR-related deaths worldwide at 4.95 million, of which 1.27 million are directly attributable to AMR [2].

The use of antibiotics in animal husbandry and agriculture is increasing. It is considered a global health problem from both animal welfare and health perspectives [3]. AMR is increasingly becoming a topic of conversation in treating infectious diseases both in Bangladesh and worldwide. Over the past 70 years, the development of effective antimicrobials has reduced the prevalence of life-threatening diseases.

However, the everyday emergence of resistance hinders this development [4].

Escherichia coli (*E. coli*) is a widespread, harmless organism for humans and animals. Most of these strains are benign commensals that coexist peacefully within the bodies of their hosts and rarely cause disease. However, *E. coli* is a very complicated species because it has evolved into pathogenic strains [5]. It has also been demonstrated that both harmful and commensal *E. coli* can act as carriers of antibiotic resistance genes, which can spread across various bacterial species, including pathogenic ones, and can transmit resistance genes to other bacteria. *E. coli* has the ability to receive and transmit resistant genes, which it then transmits to other bacteria [6-8]**.** Mobile genetic components such as plasmids and transposons enabled the rapid spread of AMR genes. This led to the emergence of drug-resistant microorganisms [9].

The enzymes known as extended-spectrum β-lactamases (ESBL) provide broad resistance to monobactams, cephalosporins, and penicillins. *E. coli* is the most common bacteria to develop resistance to beta-lactam antibiotics, but the pathogenesis is known worldwide [10]. Bacterial strains that produce ESBL may come from food animals. These strains can easily spread through the food chain. Fecal contamination can occur during processing, milking, or killing of animals and can also develop during transport and storage of the product [11]. With over 160 million inhabitants, Bangladesh is a densely populated agricultural country and the majority of people live near animals [12].

In Bangladesh, livestock farming is one of the fastest-growing sectors. Around 2 (1.90) percent of the country's gross domestic product (GDP) comes from it. Although the livestock industry accounts for a very small part of the country's GDP, it plays an important role in meeting the country's daily need for animal protein.

AMR bacteria can easily cause damage to meat and animal products when animals are slaughtered and consumed through contaminated processing equipment or storage containers. However, the misuse of antibiotics to treat, prevent, and control animal diseases leads to increased antibiotic resistance. Therefore, the purpose of our research is to investigate the prevalence of multidrug-resistant (MDR) ESBL-producing *E. coli* in beef and sheep meat obtained from different wet marketplaces in the Sylhet division of Bangladesh.

MATERIALS AND METHODS

Ethical statement

The study was approved by the Ethical Review Committee of Sylhet Agricultural University, Sylhet 3100, Bangladesh (Approval Number. SAUEC/2017/009). In this study, samples were collected following standard animal handling and sampling procedures in accordance with the Cruelty to Animals Act 1920 (Act No. I of 1920) of the Government of the People's Republic of Bangladesh.

Isolation and identification of *E. coli*

Meat samples (n = 400) were randomly selected from cattle (n_1 = 200) and sheep (n_2 = 200) and collected from 100 different marketplaces and slaughterhouses in the Sylhet division of Bangladesh. According to the previously described method [13], all meat samples (5gm each) were processed (ground with a mortar and pestle) and transferred to the nutrient broth as a swab for bacterial multiplication by using a sterile cotton bud. The isolation and identification of *E. coli* were carried out using the previously

described method [14], which involved Gram staining, bacterial culture on various culture media for the detection of specific growth characteristics (including nutrient broth, nutrient agar, Eosin-Methylene blue agar, MacConkey's agar) and numerous other biochemical tests have been performed, such as the voges-proskauer (VP) test, the methyl red (MR) test, the citrate utilization test, the indole test and the sugar fermentation test.

Antimicrobial susceptibility test

Observing the requirements of the Clinical and Laboratory Standard Institute (CLSI2020)[14], 136 isolated *E. coli*-positive samples were tested for antibiotic susceptibilities on Mueller-Hinton agar plates employing the Kirby-Bauer technique. The antimicrobial discs used in this study were obtained from Oxoid (UK) and included the following: Cotrimoxazole (1.25/23.75µg), Ciprofloxacin (5µg), Colistin (10µg), Erythromycin (15µg), Streptomycin (10µg), and Gentamycin (10µg). The strain *E. coli* ATCC 25922 served as a reference. The effectiveness of the test was confirmed only when the *E. coli* ATCC 25922 control strain's inhibitory zone diameters fell between the performance ranges. As previously described, isolates that were resistant or intermediately resistant were treated as non-susceptible [15]. If *E. coli* was identified to be resistant to one antibiotic out of three or more distinct classes of antimicrobial medicines, it was regarded as an MDR isolate, with the exception of broad-spectrum penicillin that lacked a β-lactamase inhibitor [15].

Extended-spectrum beta-lactamase identification

Following standard protocols and CLSI standards, ESBL phenotypes were confirmed from 136 isolated positive meat samples by applying 30µg Ceftazidime, Cefotaxime, Cefoxitin, and Aztreonam spaced 15 mm apart on Mueller-Hinton agar plates [16]. In addition, the Double Disk Synergy Test (DDST) was performed to confirm the diagnosis of ESBL-producing *E. coli* as previously described [10].

Statistical analysis

Data on antibiotic resistance among *E. coli* isolates are presented as frequencies or percentages. The study was performed using a two-way analysis of variance (ANOVA) without any replication to detect significant variations in prevalence. The software GraphPad Prism (version 6; GraphPad Software Inc., USA) was used to perform these statistical analyses. P values below 0.05 are considered statistically significant.

RESULTS

Prevalence of *E. coli* in beef and sheep meat

Using staining, culture, and biochemical tests, 136 *E. coli* isolates (34%) were identified from 400 samples collected from different wet marketplaces of the Sylhet division. Of these, 90 (45%) and 46 (23%) *E. coli* were separated from beef and sheep meat, respectively (Table 1). However, the frequency was noticeably ($p = 0.004$) higher in beef than in sheep meat, whereas there were no discernible ($p = 0.114$) variations in the frequency of *E. coli* between the four districts.

Table 1. Prevalence of *E. coli* in beef and sheep meat specimens at Sylhet division.

Sample	Sylhet (n_1=50,n_2=50)	Moulvibazar (n_1=50,n_2=50)	Sunamganj (n_1=50,n_2=50)	Habiganj (n_1=50,n_2=50)	Total (n_1=200,n_2=200)	*P*-value[a]
Beef (n_1=200)	29 (58%)	20 (40%)	19 (38%)	22 (44%)	90 (45%)	**0.004**[#]
Sheep meat (n_2=200)	14 (28%	11 (22%)	10 (20%)	11 (22%)	46 (23%)	0.114[##]
Total (N=400)	43 (21.5%)	31 (15.5%)	29 (14.5%)	33 (16.5%)	136 (34%)	

n_1=number of beef sample, n_2=number of sheep meat sample.*a*= *P* values were calculated using a two-way analysis of variance (ANOVA) without replication. P-values less than 0.05 are bolded. [#] Variance between beef and sheep meat. [##] Variance among the four districts under study.

Prevalence of antimicrobial resistance

The overall prevalence of antimicrobial resistance by the disc diffusion method among the studied. *E. coli* isolates (beef and sheep meat) in the Sylhet division are given in Table 2. Each isolate from the meat sample was found to be resistant to Streptomycin (78.89%), Erythromycin (100%), and Ampicillin (100%), respectively. However, not a single *E. coli* isolate examined, either from beef or sheep meat, showed resistance to the remaining five selected antibiotics (Gentamycin, Ciprofloxacin, Colistin, Levofloxacin, and Cotrimoxazole) in the four research areas. According to the CLSI recommendation, *E. Coli* isolates exhibiting intermediate resistance to an antibiotic were regarded as resistant to that drug [16]. Moreover, in resistance patterns, no significant (p = 0.084) difference was observed in isolated samples from beef and sheep meat.

The prevalence of antibiotic resistance *E. coli* isolated from beef and sheep meat collected from four research areas is shown in Table 3. The prevalence of Ampicilin, Erythromycin, and Streptomycin Resistance *E. coli* was much greater in beef compared with sheep meat. The prevalence of these antibiotic-resistant *E. coli* showed no significant difference among the research areas.

Table 2. The overall prevalence of antimicrobial resistance among the investigated *E. coli* isolates in beef and sheep meat.

Sample	Amp	Ery	Str	Gen	Cip	Col	Lev	Cot	*P* value[a]
Beef (n_1=90)	90 (100%)	90 (100%)	51 (56.67%)	0	0	0	0	0	0.084[#]
Sheep meat (n_2=46)	46 (100%)	46 (100%)	20 (43.48%)	0	0	0	0	0	0.004[##]
Total (N=136)	136 (100%)	136 (100%)	71 (78.89%)	0	0	0	0	0	

n_1=number of *E. coli* isolates from the beef sample, n_2=number of *E. coli* isolates from sheep meat sample. Amp=Ampicilin, Ery=Erythromycin, Str=Streptomycin, Gen=Gentamycin, Cip=Ciprofloxacin, Col=Colistin, Lev=Levofloxacin, Cot=Cotrimoxazole. [a]*P* values were calculated using an unreplicated two-way analysis of variance (ANOVA). *P-values* less than 0.05 are bolded. # Variance between beef and sheep meat. ## Variance among the antibiotic-resistant genes.

Table 3. Prevalence and distribution of antibiotic-resistant *E. coli* isolates from beef and sheep meat.

Antibiotics	Sylhet		Moulvibazar		Sunamganj		Habiganj		*P* value[a]
	Beef (n1=29)	Sheep meat (n2=14)	Beef (n1=20)	Sheep meat (n2=11)	Beef (n1=19)	Sheep meat (n2=10)	Beef (n1=22)	Sheep meat (n2=11)	
Amp	29 (100%)	14 (100%)	20 (100%)	11 (100%)	19 (100%)	10 (100%)	22 (100%)	11 (100%)	0.004[♯] 0.114[♯♯]
Ery	29 (100%)	14 (100%)	20 (100%)	11 (100%)	19 (100%)	10 (100%)	22 (100%)	11 (100%)	0.004[♯] 0.114[♯♯]
Str	15 (51.72%)	6 (42.86%)	13 (65%)	5 (45.45%)	11 (57.89%)	5 (50%)	12 (54.54%)	4 (36.36%)	0.001[♯] 0.164[♯♯]

N=136. n_1=number of *E. coli* isolates from beef sample, n_2= number of *E. coli* isolates from sheep meat sample. Amp=Ampicillin, Ery=Erythromycin, Str=Streptomycin. [a]*P* values were calculated using an unreplicated two-way analysis of variance (ANOVA). *P values* less than 0.05 are bolded. [♯] Variance between beef and sheep meat. [♯♯] Variance among the four districts under study.

Prevalence of multidrug-resistant *E. coli*

The MDR was analyzed using the previously stated definition [17]. The analysis was done against three antimicrobial groups: Penicillin (Ampicillin), Aminoglycosides (Streptomycin), and Macrolides (Erythromycin). The prevalence and the distribution of 3 classes of MDR among the investigated *E. coli* isolates from beef and sheep meat in the study area are given in Table 4.

71 (52.2%) of the 136 *E. coli* isolates were found to be resistant to 3 classes of antibiotics (Ampicilin + Erythromycin + Streptomycin). However, 51 (56.66%) of the 90 isolates from beef and 20 (43.47%) of the 46 isolates from sheep meat corresponded to class 3 MDR. The prevalence of MDR *E. coli* was remarkably higher in beef than in sheep meat (*P*=0.001) whereas, the prevalence of this MDR *E. coli* did not differ significantly in the four study areas (P=0.164).

Table 4. Prevalence and distribution of 3 classes of MDR *E. coli* isolates from beef and sheep meat.

Antimicrobial agents	Type	Sylhet (n_1=29, n_2=14)	Moulvibazar (n_1=20, n_2=11)	Sunamgonj (n_1=19, n_2=10)	Habigonj (n_1=22, n_2=11)	Total (N_1=90, N_2=46)	*P* value[a]
Amp+Ery+Str	Beef	15 (51.72%)	13 (65%)	11 (57.90%)	12 (54.55%)	51 (56.66%)	0.001[♯]
	Sheep meat	6 (42.86%)	5 (45.45%)	5 (50%)	4 (36.36%)	20 (43.47%)	0.164[♯♯]
	Total MDR	21 (48.83%)	18 (58.06%	16 (55.17%)	16 (48.48%)	71 (52.2%)	

N=136. n_1=number of *E. coli* isolates from beef sample, n_2= number of *E. coli* isolates from sheep meat sample. N_1= Total number of *E. coli* isolates from beef sample, N_2= Total number of *E. coli* isolates from sheep meat sample. Amp=Ampicillin, Ery=Erythromycin, Str=Streptomycin. [a]*P* values were calculated using an unreplicated two-way analysis of variance (ANOVA). *P* values less than 0.05 are bolded. [♯] Variance between beef and sheep meat. [♯♯] Variance among the four districts under study.

Prevalence of beta-lactam antibiotic-resistant and ESBL-producing *E. coli* in beef and sheep meat

The outcome of the isolated *E. coli* resistance profile against four beta-lactam-producing antibiotics (Cefotaxime, Ceftazidime, Ceftriaxone, and Aztreonam) along with the prevalence of ESBL producers are shown in Figure 1 and Table 5. Among the total 136 isolated *E. coli* from both beef (n_1=90) and sheep (n_2=46) meat, 124 (91.17%) were resistant to Ceftazidime followed by 90 (66.17%) Aztreonam, 84 (61.76%) Cefotaxime and 72 (52.94%) Ceftriaxone (Figure 1). Therefore, *E. coli* from beef showed significantly more resistance towards all four beta-lactam antibiotics compared with isolates from

sheep meat in all of the selected districts in the research (P<0.05). However, no noticeable variation was observed in the prevalence of these beta-lactam antibiotics-resistant *E. coli* among the research areas (P > 0.05) (Table 5).

The *E. coli* isolates from specimens that showed resistance to both Cefotaxime and Ceftriaxone were considered ESBL producers according to the recommendations by the CLSI guideline [16, 18]. Overall, out of the 136 *E. Coli* isolates, 21 (15.44%) were positive for producing ESBL in the disc diffusion method (Figure 1). Among them, only 2 (4.35%) *E. coli* isolates from sheep meat showed ESBL positive whereas a significantly large number of isolates, 19 (21.11%), from beef were ESBL producers (P=0.015). However, there was no apparent difference in the prevalence of ESBL-producing *E. coli* in the study area (P > 0.05) (Table 5).

Table 5. Prevalence and distribution of ESBL-producing *E. coli* isolates from beef and sheep meat.

Anti-biotic	Type	Sylhet (n_1=29, n_2=14)	Moulvibazar (n_1=20, n_2=11)	Sunamgonj (n_1=19, n_2=10)	Habigonj (n_1=22, n_2=11)	Total (N_1=90, N_2=46)	P value[a]
Cefo	Beef	12(41.37%)	15 (75%)	17 (89.47%)	16 (72.72%)	60 (66.66%)	0.003[♯]
	Sheep	4 (28.57%)	7 (63.63%)	9 (90%)	4 (36.36%	24 (52.17%)	0.129[♯♯]
Cefta	Beef	29(100%)	19 (95%)	19 (100%)	22 (100%)	89 (98.89%)	0.013[♯]
	Sheep	8 (57.14%)	7 (63.63%)	9 (90%)	11 (100%)	35 (76.09%)	0.513[♯♯]
Ceftri	Beef	12 (41.37%)	10 (50%)	12 (63.16%	14 (63.64%)	48 (53.33%)	0.043[♯]
	Sheep	6 (42.85%)	9 (81.82%)	4 (40%)	5 (45.45%)	24 (52.17%)	0.918[♯♯]
Az	Beef	18 (62.06%)	14(70%)	11 (57.89%)	15 (68.18%)	58 (64.44%)	**0.016**[♯]
	Sheep	9 (64.28%)	6 (54.55%)	8 (80%)	9 (81.82%)	32 (69.57%)	0.298[♯♯]
[b]Cefo +Ceftri	Beef	7 (24.13%)	3 (15%)	5 (26.32%)	4 (18.18%)	19 (21.11%)	**0.015**[♯]
	Sheep	1 (7.14%)	1 (9.09%)	0	0	2 (4.35%)	0.435[♯♯]

n_1=number of *E. coli* isolates from beef sample,n_2=number of *E. coli* isolates from sheep meat sample. N_1= Total number of *E. coli* isolates from beef sample, N_2= Total number of *E. coli* isolates from sheep meat sample.Cefo = Cefotaxime, Cefta = Ceftazidime, Ceftri = Ceftriaxone, Azo=Aztreonam, Cefo +Ceftri= Cefotaxime +Ceftriaxone.[a] An unreplicated two-way analysis of variance (ANOVA) was used to calculate the P values. P values less than 0.05 are bolded. [♯] Variance between beef and sheep meat. [♯♯] Variance among the four districts under study.[b] *E. coli* isolate showing resistance to both Cefotaxime and Ceftriaxone was considered ESBL-producing in accordance with the recommendations by the Centers for Disease Control and Prevention (CDC).

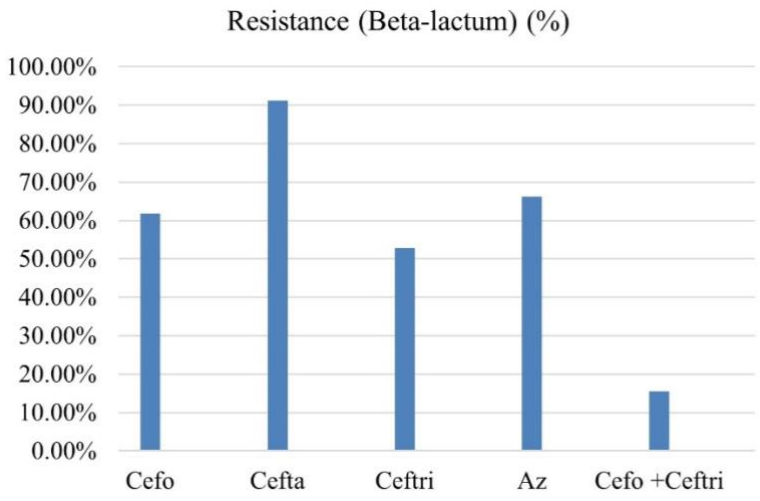

Figure 1. Overall prevalence of beta-lactam antibiotic-resistant *E. coli* isolated from beef and sheep meat. Here, the total number of isolated *E. coli* is N=136 (90 from beef and 46 from sheep meat).Cefo = Cefotaxime, Cefta = Ceftazidime, Ceftri = Ceftriaxone, Az=Aztreonam, Cefo +Ceftri= Cefotaxime +Ceftriaxone.

DISCUSSION

One of the world's fastest-growing resistance problems is caused by bacteria that produce long-spectrum lactamase [19]. Livestock could be an important means of spreading ESBL-producing bacteria throughout the community [20]. Although many AMR-related studies have been conducted with ESBL-producing *E. coli* in recent years [21], there are a few reports of the presence of these microorganisms in raw meat are available here.

According to the results of the present study, the overall prevalence of contamination of beef and sheep meat with *E. coli* was 45% and 23%, respectively. Studies in South Africa and Ethiopia reported 34.3% *E. coli* in beef [22] and 23.3%[23], 26.6 %[24], and 20.3 % [25] *E. coli* in sheep meat, which are closely similar to the current findings. Moreover, a comparatively higher prevalence of *E. coli* in beef was reported in Poland (74.5%) [26], Vietnam (74.5%) [27], Ghana (86.67%) [28] and Bangladesh (70%) [29]. For sheep meat, it was 40% [30] and 30.97% [31] in different studies in Ethiopia and 88.89% in Ghana [28]. There are several possible explanations for the observed variation in the prevalence between studies, including variations in the methods used for sampling and isolation, the number of animals, the research design, the period, and the antimicrobial treatment they received during the research procedure.

In our research, all *E. coli* (100%) isolated from both beef and sheep meat by disc diffusion method showed resistance to Erythromycin and Ampicillin and sensitivity towards Gentamycin, Ciprofloxacin, Colistin, Levofloxacin, and Cotrimoxazole. The result of greater resistance to Erythromycin and Ampicillin is consistent with the results of previous research [32, 33]. According to a study conducted in Addis Ababa, *E. Coli* had comparatively lower levels of resistance to erythromycin (94.2%) and ampicillin (82.3%) [23]. In this present study, the overall prevalence of Sterptomycin-resistant *E. coli* in meat samples was 78.89% where 56.67% and 43.48% were in beef and sheep meat samples, respectively. A similar study found [23] that 50% and 41.2% Streptomycin-resistant *E. coli* in the meat of beef and sheep, respectively. Nevertheless, [34] and [23] demonstrated that *E. coli* isolates were highly susceptible to Gentamycin as well as Ciprofloxacin. In another research, higher resistance to Cotrimoxazole was described [33] which is opposite to the present findings.

Nowadays, the increasing pattern of MDR traits in gram-negative bacteria reduces the effective treatment options and poses a great threat to both veterinary and human treatments. In our work, the total rate of MDR *E. coli* in isolated meat samples was detected at 52.2% where 56.66% were from beef samples and 43.47% from sheep meat samples. Similar findings were found in [29] (57.14% of MDR isolates from beef) and [23] (41.48% of MDR isolates from sheep meat). In some other studies, comparatively higher MDR *E. coli*, 73.4% [23] , and 71%, [35], was observed among different meat samples. However, a lower percentage of MDR isolates, 7.7% in ground beef [36] and 20% in sheep meat [33] was reported. The variation in such findings suggests a possible connection of antimicrobial agents with the development of antibiotic resistance [37, 38].

Recent studies show that the incidence of ESBL-producing Enterobacteriaceae has increased rapidly worldwide [39] and has been identified and separated from many community sources such as lettuce, raw milk, piglets, poultry, and cattle [40]. The current investigation has demonstrated that 15.44% of *E. coli* strains were ESBL-producing where 21.11% from beef and 4.35% from sheep meat were detected. This finding closely resembles a previous study [41] in which they found 7 ESBL-positive *E. coli* among 33 (21%). A relatively lower prevalence [42] carried out in Turkey and it was only 7%. According to many studies, only 4.7% and 9% of beef samples in the

Netherlands and Spain, respectively, were found to be ESBL-positive [43]. In Mexico, 40% ESBL-producing *E. coli* was observed in beef specimens [44]. Observed variations may occur due to differences in housing and feeding management, hygienic condition of drinking water, population density, and sanitary condition of slaughterhouses and processing units. PCR was not performed in our study due to a lack of funding. But the data are authentic.

CONCLUSIONS

The current investigation revealed that antibiotic-resistant ESBL-producing *E.coli* is present in significant levels in meat samples of slaughtered cattle and sheep originating from different districts of Sylhet division, Bangladesh (Figure 2). In these locations, the overall prevalence of *E. coli* was determined to be 34%. All isolated meat samples were resistant to Ampicillin and Erythromycin as well as sensitive to Gentamycin, Ciprofloxacin, Colistin, Levofloxacin, and Cotrimoxazole. In addition, 52.2% and 15.44% of the isolates were reported as 3 classes of MDR and ESBL producers respectively. The increased prevalence of MDR ESBL-producing *E. coli*, which is present in food specimens, suggests a higher danger to public health. This research recommends conducting molecular studies on antimicrobial resistance genes, virulence factors, and the genetic evolution of drug-resistant organisms to address this impending global threat.

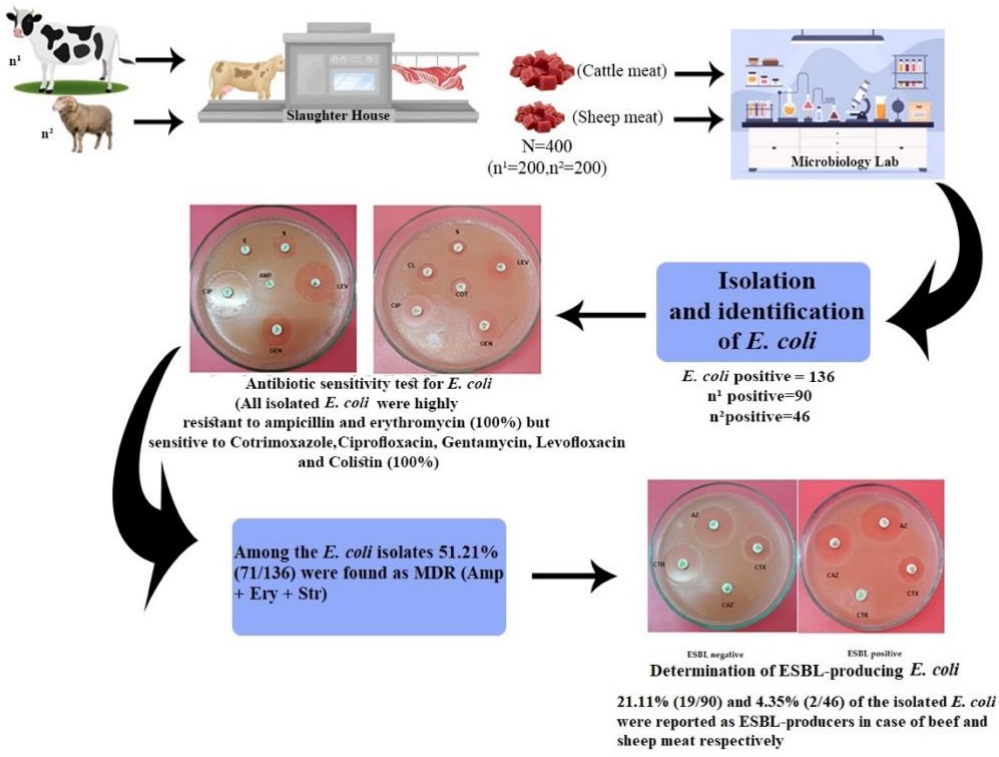

Figure 2. Samples (136) were found as *E. coli*-positive from 400 meat samples (200 cattle meat and 200 sheep meat). All of the isolated samples were highly resistant to Ampicillin and Erythromycin but sensitive to Cotrimoxazole, Ciprofloxacin, and Gentamycin. In addition, 71 (out of 136) were found to MDR (Amp+Ery+Str). ESBL-producing *E. coli*, which showed resistance to both Cefotaxime and Ceftriaxone, was found 21.11%.

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AUTHOR CONTRIBUTIONS

Conceptualization: NSR, MAZ, BP, and MAR; methodology, NSR, SY, AH, and NYR; software, NSR, AH, NYR, MSI, MAL, and CAS; validation, NSR, and MAR; formal analysis, NSR, SY, and BP; investigation, NSR, NYR, and CAS; resources, MMR; data curation, NSR; writing—original draft preparation, NSR, and MAR; writing—review and editing, AH, MSS, MTH, MAS, MP, MAZ, and BP; supervision, MMR. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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