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Research Article

Investigating the presence of multidrug-resistant *Escherichia coli* in raw mutton from open marketplaces

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A B S T R A C T

The objective of this research was to identify antibiotic-resistant strains of *Escherichia coli* and examine the occurrence of *E. coli* in mutton samples sourced from multiple locations within two districts, given the serious public health risks associated with its presence in food. Six mutton samples were procured from open markets in Kushtia, while another six were sourced from markets in Jhenaidah. Serial dilutions were made by processing mutton after samples were gathered from every region. The samples were plated on nutrient agar and cultured for bacterial growth using the spread plate technique. Following incubation, bacterial contamination was evident in every sample, ranging from 1.2×10^9 CFU/mL to 4.7×10^9 CFU/mL. After conducting selective agar tests (MacConkey agar and Eosin-methylene blue) and biochemical tests (Methyl Red, Catalase, Citrate, Oxidase, Indole, and Voges-Proskauer test) to verify the presence of *E. coli*, it was discovered that 66.67% of the samples of mutton were positive for the *E. coli*. Then, the *E. coli* strains were subjected to additional investigation, which showed resistance to three (Amoxicillin, 30 µg; Penicillin, 10 µg; and Co-trimoxazole, 25 µg) of the eight tested antibiotics and another five (Ciprofloxacin, 5 µg; Azithromycin, 30µg; Streptomycin, 10 µg; Levofloxacin, 5µg; and Erythromycin, 15µg) antibiotics were sensitive. The study underscores the possibility of antibiotic-resistant and multidrug-resistant *E. coli* in mutton samples, underscoring the need for appropriate handling and cooking techniques to prevent foodborne illnesses from mutton contamination.

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INTRODUCTION

Meat provides various nutritional benefits, such as being an exceptional source of protein, abundant in energy, and containing a high concentration of vitamins and minerals (Webb *et al.*, 2005; Casey *et al.*, 2005; Mazhangara *et al.*, 2019; Chivandi *et al.*, 2019; Adzitey *et al.*, 2020). In addition to serving as a concentrated source of energy, meat's lipids also facilitate the absorption of fat-soluble vitamins, reducing the risk of a possible vitamin deficit (Mazhangara *et al.*, 2019). However, Bacterial spoilage of meat relies on the initial number of microorganisms, the time and temperature of storage conditions, and the meat's physicochemical attributes (Doulgeraki *et al.*, 2012). Global public health is also at risk due to the emergence of antibiotic-resistant foodborne bacteria, including *Escherichia coli*, *Salmonella*, and *Listeria monocytogenes* in meat (Laury *et al.*, 2009). The main source of contamination in slaughterhouses is improper handling and hygienic conditions (Bakhtiary *et al.*, 2016). *Escherichia coli*, also known as *E. coli*, is an *Enterobacteriaceae* family member (Addis and Sisay, 2015), one of the frequently isolated foodborne pathogens from animal flesh and meat-based items (Davis *et al.*, 2018). *E. coli* is easily spread via the food chain in various sources, including domestic and wild animals (Rahman *et al.*, 2024) and may be cross-contamination linked to a variety of acute and invasive human diseases (Addis and Sisay, 2015). It is a very adaptable type of bacteria that can cause problems in the intestines or outside the body (Hammerum and Heuer, 2009). It includes both pathogenic and nonpathogenic strains (Hammerum and Heuer, 2009). There are strains of *E. coli* that are derived from meat that can be opportunistic and pathogenic, but the majority of these strains are either commensal pathogens in humans or they are part of the

native flora and contribute to the important functions carried out by the intestinal microflora (Hammerum and Heuer, 2009). *E. coli* often contaminates meat through handling, incorrect dressing, cleaning, and unsanitary selling procedures. *E. coli* infections are thought to be possible from contaminated meat, either through direct contact with the bacteria during food preparation or through ingestion of raw or undercooked meat products (Addis and Sisay, 2015). While *E. coli* is sensitive to heat in the 60–80 ºC range, other strains of the bacteria have been shown to be extremely heat resistant (Li and Gänzle, 2016). It has been documented that adding salt can make a number of *E. coli* strains resistant to heat and that 2% of *E. coli* isolates, including food isolates, carry genes that make them more resistant to heat (Garcia-Hernandez *et al.,* 2015). By means of lateral gene transfer, one *E. coli* strain can acquire the locus of heat resistance from another (Mercer *et al.*, 2015). Additionally, the length of the microwave exposure and the cooking techniques employed may cause *E. coli's* thermal inactivation to fail (Göksoy *et al*., 2000). Through various processes, *E. coli* can also withstand low-temperature stress, sometimes known as cold shock (Yamanaka and Inouye, 2001). One of the most significant reactions to low temperatures is the production of cold-shock proteins (CSPs), which are crucial for several vital processes in *E. coli*, including transcription, translation, mRNA degradation, protein synthesis, and recombination (Chung *et al.,* 2006). Rapid freezing, on the other hand, has been shown to cause an instantaneous loss of viability in exponentially developing *E. coli* cells of up to 90% (Cao-Hoang *et al.,* 2008). However, abdominal pains, vomiting, and occasionally bloody diarrhea are the most typical signs of *E. coli*-caused food poisoning in humans (Switaj *et al.* 2015). An infection with *E. coli* that produces Shiga toxin can occasionally result in hemolytic-uremic syndrome, which can induce renal failure (Switaj *et al.,* 2015). The majority of *E. coli* infections resolve on their own; thus, taking antibiotics is not advised (Wong *et al.,* 2000). They pose a serious threat to human health if they lead to antibiotic resistance due to frequent use or other reasons.

Antimicrobials are typically used to treat microbial illnesses in people and animals (Hoelzer *et al.,* 2017). Antimicrobial use in farm animal treatment has been associated with the emergence of multidrug-resistant bacteria, posing a risk to public health (Mouiche *et al.*, 2019). Different resistances to various antimicrobials, such as erythromycin, tetracycline, ampicillin, gentamicin, sulphamethoxazole/trimethoprim, chloramphenicol, cefuroxime and ceftriaxone, have been observed in *E. coli* isolated from red meat samples (Aslam and Service, 2006; Adzitey, 2015; Anning *et al.*, 2019; Saud *et al.*, 2019).

The aim of this research is to analyze the occurrence and antibiotic resistance patterns of *E. coli* isolated from uncooked mutton samples collected from open markets in Kushtia and Jhenaidah. This study aims to shed light on the possible consequences for public health and guide future regulations for the use of antibiotics in animal production by examining the patterns of resistance and pinpointing the particular drugs to which these bacteria are resistant. The results will support initiatives to guarantee food safety and public health protection as well as add to the expanding body of information on antibiotic resistance.

MATERIALS AND METHODS

Collection of samples

Mutton samples were gathered from open markets in 2 different cities in Bangladesh. We collected six samples from six different locations of Kushtia districts and another six samples were collected from different locations of Jhenaidah districts. Mutton was collected under sterile conditions and stored in a sanitized plastic bag. Then transfered to the Microbiology Laboratory, Islamic University, Kushtia-7003 using an icebox within one hour. The samples were stored as soon as possible stored at -17ºC maintaining all the biosafety rules of the institution.

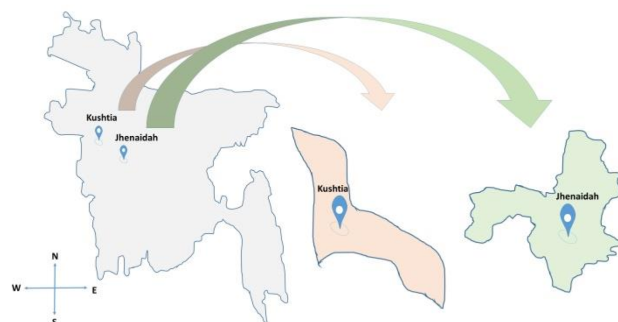


Figure 1. The frame of Bangladeshi Map was taken from Google earth (https://www.google.com/earth/about/). Major study areas (Kushtia and Jhenaidah) were marked by distinct icons.

Sample preparation

The samples were sliced into small, thin pieces using a disinfected knife and subsequently ground with a mortar and pestle under aseptic conditions. The analytical portions were transferred to another sterile conical flask and 100 ml of distilled water was added. The solutions were vigorously agitated using a shaking apparatus, and the water from the samples was collected.

Total bacterial count

The total bacterial count was determined for all samples using the Lazy Susan plating technique on solid Nutrient agar plates. The cultures were used to prepare bacteria inoculum, which was standardized to 10-fold CFU/mL by serial dilution. The nutrient medium was autoclaved and maintained at 46ºC. Then, 1 ml diluted suspension was taken for spreading culture and to ensure even distribution of inoculum. The plates were placed in an incubator at 37ºC overnight. The plate counting method was used to determine the colony-forming unit.

Isolation of *E. coli* from the mutton sample through selective media

MacConkey (MAC) agar media

For the preparation of MAC agar, we autoclaved the agar media. Then in laminar airflow, we poured the media into the petri dish when it came to 46ºC. Bacteria were picked by a sterile inoculating loop from the nutrient agar plate and

streaked on the MAC media. Incubated overnight and observed the morphology of the bacteria.

Eosin Methylene Blue (EMB) agar media

Nutrient agar media was used for bacterial growth before. After overnight incubation bacterial colony appeared. EMB agar media was autoclaved and in an aseptic condition, a pure single colony was picked up from the plate and streaked gradually on the EMB agar plate and in an incubator for 48h at 37ºC. The plate was observed after overnight incubation.

Biochemical test for the identification of *E. coli*

Gram staining

A drop of distilled water was taken onto a sterilized slide. A pure colony was picked with a loop, mixed with the bacteria with the water, and waited until the sample was air-dried. To fix the bacteria the slide was slightly heated and then cooled. The bacteria were exposed to Crystal Violate for 1 min and washed with distilled water. Further, iodine solution was applied for 30 sec to 1 minute, flooded with distilled water, decolorized with an acetone alcohol solution, and washed immediately with distilled water so that the bacteria were not over-decolorized. Next, safranin was applied for 1 minute and rinsed properly. Finally, the slide was examined under a phase contrast fluorescence microscope.

Citrate test

Simmons citrate agar was used to perform the citrate test. A slant was prepared into a screw caps test tube and fresh (16-18h) pure cultured bacteria were inoculated there, incubated for 18-24h at 37ºC, and observed the color for citrate test result.

Oxidase test

1% of tetramethyl-p-phenylene-diamine dihydrochloride was freshly prepared and Whatman's No. 1 filter paper was soaked for 30 seconds in a petri dish. With a sterilized loop, a pure colony was transferred and spread onto the paper. After 1 min we observed the result of the oxidase test.

Indole test

A tiny amount of pure bacterial culture was introduced into tryptophan broth and subsequently incubated at 37 ºC for a period ranging from 24 to 48 hours. Then during the incubation period, 5.0 to 10.0 drops of Kovac's reagent were gradually introduced into the tube. The results were observed within 2 minutes.

Methyl Red – Voges Proskauer (MR-VP) test

In the methyl Red test, a pure bacterial culture colony was inoculated into MR-VP broth and left to incubate at 37 ºC for 24-48 hours. Afterward, a small quantity of Methyl-Red reagent was added drop by drop. We waited for 15 minutes to detect any color change. In the Voges-Proskauer Test, isolated bacteria were cultured overnight at 37 ºC in MR-VP broth. The next day, a few drops of VP1 reagent or 5% Alpha-naphthol was added to the culture test tube and shaken properly. After that, VP 2 reagent or 40% KOH was added 5-10 drops. Waited 15-20 minutes for the result.

Catalase test

Our targeted bacteria were cultured overnight. 3% hydrogen peroxide (H_2O_2) was taken on a sterile slide. A pure colony of our cultured bacteria was mixed with it, and we waited for 1 minute for the result.

Antibiotic susceptibility test

The Antibiotic sensitivity test was performed to identify the effect of antibiotics against the bacteria found in meat. Bacterial samples from pure culture were taken in sterile cotton buds and carefully spread into the nutrient agar. The different antibiotic disk namely Ciprofloxacin (5 µg), Azithromycin (30µg), Amoxicillin (30 µg), Streptomycin (10 µg), Levofloxacin (5µg), Erythromycin (15µg), Penicillin (10 µg), and Co-trimoxazole (25 µg).

Antibiotic disks were placed into the agar medium under aseptic conditions. Antibiotic susceptibility can vary among organisms, even within different strains. Hence, a range of susceptibility criteria is employed to determine whether an organism is susceptible, resistant, or exhibits intermediate resistance to the specific antibiotic. Muller Hinton Agar was utilized for the lawn culture of tested bacteria, following the Kirby-Bauer method. The cultured plates were then incubated overnight at 37 ºC to obtain the results.

RESULTS

Total bacterial load of meat samples

The number of colony-forming units was enumerated after a 24-hour period and expressed as CFU/mL. Six samples (1-6) were collected from the Kushtia district and six samples (7-12) were collected from Jhenaidah. Those were cultured in a nutrient agar medium, where sample 10 (Fig 2) showed the highest contamination. We found that the maximum bacterial load was 4.7×10^9 CFU/mL. On the contrary, we observed the lowest contamination (1.2×10^9 CFU/mL) in sample 6. Therefore, this result suggests that all the mutton samples collected from the Kushtia and Jhenaidah districts of Bangladesh have bacterial contamination.

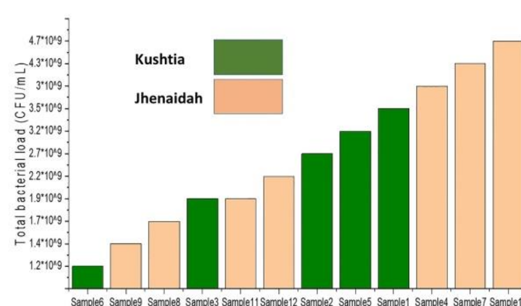


Figure 2. Graphical presentation of total bacterial load on six mutton samples collected from the Kushtia district (shaded by green color) and another six mutton samples from the Jhenaidah district (shaded by light yellow color). Total bacterial load was expressed in CFU/mL).

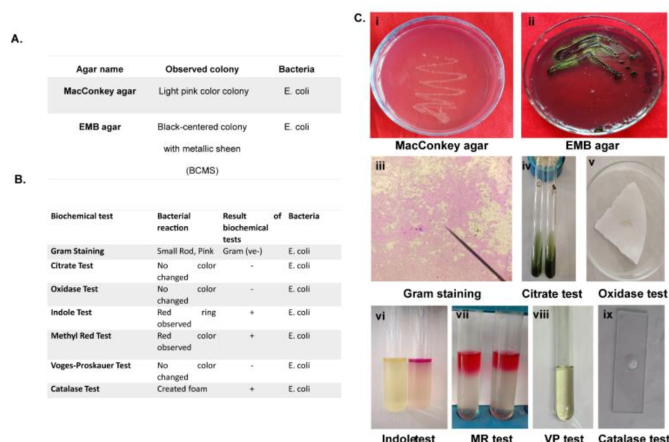
Isolation and characterization of *E. coli* from mutton samples

To identify the isolated colony of bacteria, selective agar and biochemical tests were performed. The growth pattern and morphology of bacteria on MacConkey agar and EMB agar and some biochemical tests (Methyl Red, Indole, Catalase, Citrate, Voges-Proskauer, and Oxidase Test) results confirmed the presence of *E. coli*. Selective agar and

biochemical tests confirmed the presence of *E. coli* in 8 samples among 12 samples. So, among all the mutton samples, 66.67% carried *E. coli.*

Isolation and characterization by selective agar media for *E. coli*

Sorbitol-MacConkey (MAC) Agar media selectively supports the growth of *E. coli*, which are gram-negative, non-fastidious rods. When *E. coli* ferments lactose, it appears as red or pink colonies on the agar(Allen, 2005). Among 12 isolated bacteria, 8 isolated bacteria are gram-negative non-fastidious rods. They fermented lactose and appeared light pink on agar after overnight incubation (Fig. 3A, Ci). The other 2 samples showed white-colored colonies, and another 2 samples did not grow on this selective agar medium. For the isolation of *E. coli,* we used EMB agar where the green metallic sheen of bacteria confirms the presence of *E. coli* (Divya *et al.*, 2016). Our isolated bacteria were given on EMB agar and after overnight incubation, 8 samples gave a metallic green sheen (Fig. 3A, Cii) and the other 4 samples did not grow. So, the selective agar tests proved that our isolated 8 samples were *E. coli*.


A.

Agar name	Observed colony	Bacteria
MacConkey agar	Light pink color colony	E. coli
EMB agar	Black-centered colony with metallic sheen (BCMS)	E. coli

B.

Biochemical test	Bacterial reaction	Result of biochemical tests	Bacteria
Gram Staining	Small Rod, Pink	Gram (ve-)	E. coli
Citrate Test	No color changed	-	E. coli
Oxidase Test	No color changed	-	E. coli
Indole Test	Red ring observed	+	E. coli
Methyl Red Test	Red color observed	+	E. coli
Voges-Proskauer Test	No color changed	-	E. coli
Catalase Test	Created foam	+	E. coli

Figure 3. Biochemical tests are done to identify *Escherichia coli*. **(A)** *E. coli* are identified by selective agar test **(B)** *E. coli* are identified by biochemical tests **(C)** Observations of different selective agar and biochemical tests to identify *E. coli.*

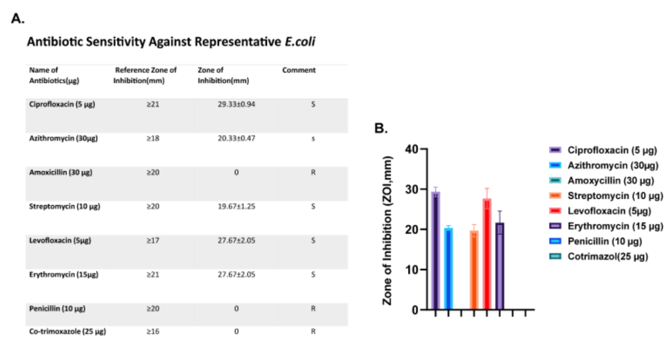
Isolation and Characterization of *E. coli* by biochemical test

Gram-negative bacteria, like *E. coli*, exhibit a less substantial peptidoglycan layer in their cell wall when compared to Gram-positive bacteria. This difference is what the Gram stain targets. During Gram staining, crystal violet binds to all bacteria. In Gram-negative bacteria like *E. coli,* a decolorizing agent washes away the crystal violet due to the thinner peptidoglycan layer (Beveridge, 2001). These bacteria then take up a counterstain (safranin) and appear red under the microscope in this test I observed red, rod-shaped bacteria under the microscope after a Gram stain in 8 samples, it indicates Gram-negative bacteria were presented in 8 samples (Fig. 3B, C-iii). The Citrate test assesses a bacterium's ability to utilize citrate, a specific organic acid, as its sole carbon and energy source. *E. coli* often has the enzymes necessary to break down and utilize citrate. So, a positive Citrate test result increases the likelihood of *E. coli* being present (Reiner, 2010). Our 8 samples gave a positive result in the citrate test (Fig. 3B, Civ). The Oxidase test checks for the presence of an enzyme called cytochrome c oxidase. This enzyme helps some bacteria use oxygen in their respiratory process. *E. coli* generally doesn't possess cytochrome c oxidase. Therefore, it won't oxidize the reagent in the Oxidase test, leading to a negative result (Hemraj *et al.,* 2013). In our oxidase test we observed no color change in 8 samples (Fig. 3B, C-v). The Indole test employs a reagent that undergoes a color change (typically to red) in the presence of indole, indicative of E. coli presence (Wang *et al.*, 2001). In my study I observed red rings in 8 samples (Fig. 3B, C-vi). Methyl red (MR) test assesses the type of acidic end products produced by bacteria during glucose fermentation. *E. coli* ferments glucose through a pathway called mixed-acid fermentation (Hemraj *et al.*, 2013). So, color change causes. We observed red color in 8 samples (Fig. 3B, Cvii). *E. coli* typically produces a negative Voges-Proskauer (VP) test result (no color change) (Abdallah *et al.*, 2016). We observed 8 samples, where there was no color change (Fig. 3B, C-viii). *E. coli* creates gas bubbles in the catalase test (Hemraj *et al.*, 2013). We observed gas bubbles in 8 samples (Fig. 3B, C-ix).

Isolated *E. coli* showed resistance against antibiotics

As 66.67% samples were contaminated by *E. coli*, I took one of them as a representative *E. coli*. The disc diffusion technique was used for the antibiotic sensitivity test. The susceptibility of the isolated *E. coli* was evaluated against a diverse array of antibiotics to detect multidrug-resistant bacteria.

The following antimicrobial disks with their corresponding Ciprofloxacin (5µg), Penicillin (10µg), Azithromycin (30µg), Amoxicillin (30µg), Streptomycin (10µg), Levofloxacin (5µg), Erythromycin (15µg), and Co-trimoxazole (25µg). In figure 3, the inhibition zone was reported as the size of the inhibition zone surrounding the individual disk which summarizes the antibiotic resistance profile of the bacterial isolate in this study. According to our study, *E. coli* is sensitive against Ciprofloxacin, Azithromycin, Streptomycin, Levofloxacin, and Erythromycin antibiotics (Fig. 4A, B). These antibiotics gave 29.33±0.94, 20.33±0.47, 19.67±1.25, 27.67±2.05, 27.67±2.05 mm zone of inhibition gradually. Besides, Amoxicillin (30 µg), Penicillin (10 µg) and Co-trimoxazole (25 µg) were resistant to *E. coli*. No clear zone was observed (Fig. 4A, B) which indicates that the isolated *E. coli* from raw mutton samples were multidrug resistant.


A.

Antibiotic Sensitivity Against Representative *E.coli*

Name of Antibiotics(µg)	Reference Zone of Inhibition(mm)	Zone of Inhibition(mm)	Comment
Ciprofloxacin (5 µg)	≥21	29.33±0.94	S
Azithromycin (30µg)	≥18	20.33±0.47	s
Amoxicillin (30 µg)	≥20	0	R
Streptomycin (10 µg)	≥20	19.67±1.25	S
Levofloxacin (5µg)	≥17	27.67±2.05	S
Erythromycin (15µg)	≥21	27.67±2.05	S
Penicillin (10 µg)	≥20	0	R
Co-trimoxazole (25 µg)	≥16	0	R

Figure 4. Antibiotic sensitivity against isolated *Escherichia coli.* **(A)** Determination of zone of inhibition and data presented as median ± standard deviation (M±SD). **(B)** Graphical presentation of antibiotic sensitivity against isolated *E. coli.*

DISCUSSIONS

The purpose of this study was to find out how common *E. coli* was in samples of mutton from some local markets in Kushtia and Jhenaidah districts. The findings indicate that *E. coli* was detected in 66.67% of the tested samples (8 out of 12), which may raise serious concerns about public health because *E. coli* is the main cause of travelers' diarrhea and infant diarrhea considering the elevated prevalence of *E. coli* in these samples, it is crucial to ensure proper handling and processing of mutton products to prevent the transmission of foodborne infections (Arvanitoyannis *et al.,* 2009). The biochemical tests employed in this investigation verified that *E. coli* was present in the samples (Beveridge, 2001; Wang *et al.,* 2001; Reiner, 2010; Hemraj *et al.,* 2013; Abdallah *et al.,* 2016), which is in line with other studies on the occurrence of *E. coli* in meat products (Ahmed *et al.,* 1995; Bolton *et al.,* 1996; Schroeder *et al.,* 2004; Sofos and Georgaras, 2010). Three of the eight *E. coli* isolates tested positive for antibiotic resistance, highlighting the significance of keeping an eye on antibiotic resistance in microorganisms. This finding is particularly alarming and points to the increasing problem of antibiotic resistance in foodborne pathogens. The presence of antibiotic-resistant *E. coli* in food products poses a severe public health risk (Osman *et al.,* 2018). When antibiotic-resistant bacteria enter the human food chain, they can cause infections that are difficult to treat (Perreten, 2005). This can lead to increased morbidity, longer duration of illness, and higher medical costs (De Kraker *et al.,* 2011; Naylor *et al.,* 2019; Daneman *et al.,* 2023). The resistance observed in this study underscores the urgency of addressing antibiotic usage in livestock. Few studies were conducted worldwide that concentrated on the microbiological quality of mutton samples; these studies are noteworthy (Sheridan *et al.,* 2003; Goetsch *et al.,* 2011; Pophiwa *et al.,* 2020). This study offers important insights into possible dangers related to mutton eating in Bangladesh, where mutton consumption is common. The results imply that, in order to lower the danger of *E. coli* infection, better handling and processing procedures may be required in the mutton business. The study's findings have effects on public health education and policy as well. The significant presence of *E. coli* in mutton samples emphasizes how crucial it is for consumers to handle and prepare food properly. The report also emphasizes the necessity of raising public knowledge of the dangers of consuming tainted meat products. By examining the genetic variety of *E. coli* isolates from mutton samples and looking into possible sources of contamination in the mutton business.

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, this study not only demonstrates the occurrence of *E. coli* in mutton specimens from two areas but also reveals the presence of antibiotic-resistant strains. It emphasizes the necessity of monitoring the microbiological standards of mutton specimens in Kushtia and Jhenaidah, highlighting the need for enhanced handling and processing practices to minimize the risk of *E. coli* contamination. Urgent actions are necessary to enhance food safety protocols and to regulate antibiotic usage in meat handling. Future studies could build on these findings. Additionally, research on the efficiency of various handling and processing techniques in lowering *E. coli* contamination and antimicrobial-resistant strains may offer insightful information for creating of successful public health initiatives.

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