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Screening for *Escherichia coli* in Chopping Board Meat Samples and Survey for Sanitary and Hygienic Practices in Retail Meat Shops of Bharatpur Metropolitan City, Nepal

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Abstract: In this study, chopping board meat samples collected from meat shops of Bharatpur Metropolitan City, Nepal, were screened for the presence of *Escherichia coli* (*E. coli*), with a special emphasis on the identification of extended-spectrum β-lactamase (ESBL)-producing strains. Representatives from the meat shops were also interviewed to understand the sanitary status and hygienic practices. *E. coli* bacteria were detected in one third (33/99) of the meat samples, while none of the samples had ESBL-producing strains. While 60.6% (60/99) of the meat shop personnel wore protective clothing, 15.15% (15/99) used gloves, and only 5.05% (5/99) had separate equipment for cleaning the viscera of animals. This study highlights the need for the regular screening of meat samples to identify pathogenic bacteria such as *E. coli* and for improvements in the sanitary status and hygienic practices of retail meat shops in Bharatpur Metropolitan City, Nepal.

Keywords: antimicrobial resistance; *Escherichia coli*; extended-spectrum β-lactamase; slaughterhouse hygiene

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1. Introduction

Antibiotics are used globally as a prophylactic measure or for the treatment of various diseases in livestock and humans. However, the injudicious use of antibiotics both in animals and in humans has led to the global emergence of antimicrobial resistance (AMR) [1]. A bulletin from the World Health Organization (WHO) in 2016 reported 700,000 deaths that year due to AMR bacteria, and the death toll was estimated to rise to 10 million annually by 2050 [2]. Due to antibiotic overuse, overprescription, nonprescription purchase, hoarding, commercial pressures, agriculture applications, and the failure of control measures to prevent the spread of resistant bacteria, multidrug-resistant bacteria have become a huge problem in human and veterinary medicine [3]. Globally, around 5 million deaths were estimated in association with AMR during 2019. While western sub-Saharan Africa had the highest level of deaths (27.3 deaths/100,000) attributable to AMR, Australia had the lowest death rate (6.5 deaths/100,000). Lower respiratory tract infection was the major disease type, while *Escherichia coli* (*E. coli*) was the leading pathogen to cause AMR-associated deaths [4].

Extended-spectrum β-lactamase (ESBL)-producing strains of Enterobacteriaceae, particularly *E. coli* and *Klebsiella pneumoniae*, have emerged as the major AMR bacterial problem in hospitalized patients [5]. *E. coli* is a Gram-negative facultative anaerobe and a rod-shaped

bacilli that inhabits the commensal and harmless intestinal microflora in different animals, birds, and humans [6]. Some strains of *E. coli*, however, are pathogenic and can cause intestinal or extraintestinal diseases in animals, birds, and humans. Animals and birds can serve as reservoirs for pathogenic strains of *E. coli*, and zoonotic transmission is plausible [6]. For example, cattle are the well-known reservoir for the Shiga-toxin-producing enterohemorrhagic *E. coli* (EHEC) strain O157:H7, which causes over 63,000 annual hemorrhagic colitis cases in the United States alone [7]. Besides cattle, antibiotic-resistant *E. coli* O157:H7 has also been isolated from raw sheep and goat meats, indicating their potential role in human transmission [8]. Similarly, avian pathogenic *E. coli* (APEC) was also indicated to have zoonotic potential [9]. APEC is an extraintestinal pathogenic *E. coli* (ExPEC) that causes colibacillosis in avian species and can result in up to 53.5% mortality in young chickens [9,10]. APEC isolates from poultry share genetic similarities with human ExPECs, bear virulence factors relevant to human uropathogenic *E. coli* and neonatal meningitis *E. coli*, and cause urinary tract infections (UTIs) and meningitis in rodent experiments, indicating that they are a potential source for foodborne *E. coli* transmission [9,10].

In Nepal, livestock farming, especially the goat and poultry sectors, is expanding rapidly, together with the consumption of meat, milk, and eggs [11,12]. While there was a yearly increment of 3.74% in the goat population in Nepal between 2008/9 and 2018/19, the chicken population increased tremendously by 19.5% per year [12]. In parallel, the use of antibiotics increased, as shown by a 50% increment in the antibiotics use in the veterinary sector between 2008 and 2012 [13]. Advancements in the field of livestock and the poultry sector further enhanced the use of antimicrobials as a growth promoter in feed or as a medication [13]. Over nine classes of antimicrobials, including cephalosporins, aminoglycosides, and fluoroquinolones, were used during 2019 in the livestock sector in Nepal, and the total quantity of antimicrobials used was over 47,000 Kg [14].

The use of antimicrobials is not well-regulated in Nepal, and the self-prescription, over or underprescription, and irrational use of antimicrobials are highly prevalent in the animal and poultry sectors, which contribute significantly to the growing issue of AMR [13]. An earlier study carried out in Chitwan, Nepal, showed that 94% of the isolates of *E. coli* from colibacillosis-suspected broiler chickens were resistant to three or more antimicrobials [15]. This raises concern over the possibility of the emergence and dissemination of AMR bacteria through animal products, including meat. This study was carried out in retail meat shops of Bharatpur Metropolitan City of Chitwan, Nepal, to determine the prevalence of *E. coli* in meats dispatched from the meat shops, using chopping board meat samples. Further emphasis was given to detecting ESBL-producing *E. coli* and to determine the sanitary and hygienic status of the meat shops.

2. Materials and Methods

2.1. Sample Collection

As per the Cottage and Small Industries Office of Chitwan District, there were a total of 140 meat shops registered to sell meat in 2019. We collected samples from 99 of those retail meat shops from the main areas of Bharatpur Metropolitan City, including Narayangarh, Pokhara Bus Park, Hakim Chowk, and Rampur. The laboratory work and tests were performed in the Veterinary Microbiology and Parasitology Laboratory of the Agriculture and Forestry University, Rampur, Nepal. In Nepal, meats are mostly dispatched to consumers after chopping into smaller pieces. All these meat shops sold chicken and goat meat and used common chopping boards to cut meat into pieces. Meat samples were collected in sterile sample boxes by extracting the leftovers from the chopping boards. Meat samples were stored in an icebox for preservation and were carried to the laboratory.

2.2. Identification of Bacteria

In the laboratory, 25 gm meat samples were weighed and added to 225 mL of buffered peptone water and were then incubated aerobically overnight at 37 °C. After incubation, the sample and peptone water mixes were stirred using sterile cotton buds. The buds

were pressed against the wall of the container to remove excess fluid and then spread in MacConkey agar plates [16]. After incubation at 37 °C for a day (18–24 h), the bacterial colonies were identified, and suspected colonies were transferred to Eosin-Methylene Blue (EMB) agar using an inoculation loop for the confirmation of *E. coli* [17]. After a day (18–24 h) of incubation, colonies with a metallic sheen were identified as *E. coli*-positive colonies. These colonies were used for two purposes: first, for biochemical tests to further verify them as *E. coli*, and second, for antibiotic sensitivity testing to determine the ESBL-producing strains of *E. coli*. The plates were stored at 4 °C for up to 7 days before being confirmed with the biochemical tests. The biochemical tests were those that are commonly employed for the identification of Enterobacteriaceae, viz. the indole, methyl red, Voges–Proskauer, citrate utilization (IMViC), and oxidase tests [18–21]. All these procedures were carried out following the guidelines of Clinical and Laboratory Standard Institute (CLSI) [22].

2.3. Screening of ESBL-Producing Strains of E. coli

For antibiotic sensitivity testing, 3–4 bacterial colonies were taken from an EMB agar plate and transferred into 5 mL of 0.85% sterile saline, and the turbidity of the sample was adjusted to a matching 0.5 McFarland standard at 550 nm using a densitometer (SIA Biosan, Riga, Latvia). The bacterial concentration of the sample was adjusted either by adding bacterial colonies or by adding sterile water to the sample. To screen for ESBL-producing strains of *E. coli*, an ESBL kit (MAST Group Ltd., UK) was used. Briefly, positive samples were suspended uniformly over the entire area of a Mueller–Hinton susceptibility agar plate. Samples were then tested with a clavulanic acid (CV) and antibiotics (i.e., Cefotaxime (CTX-30mcg) and Ceftazidime (CAZ-30mcg)) combination using a standard disc diffusion procedure, as suggested in the CLSI guidelines [22]. The plates were incubated at 35 °C ± 2 °C in the presence of ambient air for 16–18 h. The zone of inhibition was measured using a sliding caliper and was interpreted by comparing with a Kirby–Bauer chart. The CLSI suggests ≤27 mm and ≤22 mm as the normal zones of inhibition exhibited by Cefotaxime and Ceftazidime, respectively, in the absence of beta lactamase inhibitors. A difference of ≥5 mm between the zones of the diameters measured with and without using beta lactamase inhibitors indicated an ESBL-producing strain of *E coli* in the tested sample [22].

2.4. Questionnaire Survey of Retail Meat Shop Representative

Representatives of all 99 meat shops were interviewed during a visit between March and May 2020 using a set of questionnaires, and meat shops were inspected regarding hygienic and sanitary practices (Appendix A).

2.5. Data Analysis

Data were entered into MS Excel (version: professional plus 2013) and analyzed using descriptive statistics. The presence of ESBL-producing *E. coli* was determined by calculating the differences in the diameters of the zones of inhibition after using the antibiotics CTX-30 and CAZ-30 with or without CV [23]. The survey results are also presented as frequencies.

3. Results

3.1. Identification of E. coli

Out of the 99 tested samples, 52.53% (52/99) of the samples showed colony characteristics indicative of *E. coli* (Table 1). One colony from each suspected positive sample was further transferred to selective and differential media for *E. coli*, i.e., the EMB agar. Among the 52 suspected samples, 33 samples had colonies that produced a characteristic metallic green sheen. Colonies from those 33 samples were further confirmed with biochemical tests (Table 1). Thus, a total of 33.33% (33/99) of the samples were positive for *E. coli*.

Table 1. Results of cultures and tests performed to identify *E. coli* in chopping board meat samples.

Serial Number	Tests Performed	Results (Positive/Total Samples)	Inferences
1	MacConkey agar	52/99 (52.53%)	Colony characteristics Off-white with shiny mucous texture all over the plate Slightly raised colony
2	Eosin-methylene blue agar	33/99 (33.33%)	Metallic green sheen
Biochemical tests			
1	Indole test	33/33 (100%)	Red layer at the top of the tube
2	Methyl red test	33/33 (100%)	Red color after the addition of methyl red reagent
3	Voges–Proskauer test	33/33 (100%)	Lack of color change
4	Citrate utilization test	33/33 (100%)	Lack of growth and color change in the tube

To determine whether there were any ESBL-producing *E. coli*, colonies from 33 positive samples were tested for their susceptibility to two beta-lactam antibiotics: CTX 30mcg and CAZ 30mcg. The diameters of the zones of inhibition between the antibiotics tested alone or in combination with clavulanic acid were determined, and no significant differences (i.e., $\geq$5 mm) were observed (Table 2). Thus, we did not detect the presence of ESBL-producing *E. coli* in these meat samples.

Table 2. Zones of inhibition of CTX and CAZ antibiotic discs tested alone and in combination with clavulanic acid.

Zone of Inhibition (Diameter in Millimeters)						
Sample Number	CTX-30 µg	CTX + CV (30 + 10) µg	Difference in Diameter	CAZ-30 µg	CAZ + CV (30 + 10) µg	Difference in Diameter
1	30	31	1	25	26	1
2	30	30	0	25	25	0
3	30	30	0	25	25	0
4	30	30	0	25	25	0
5	28	30	2	27	27	0
6	30	31	1	26	27	1
7	30	30	0	26	26	0
8	29	29	0	25	27	2
9	30	32	2	24	26	2
10	30	30	0	25	26	1
11	30	30	0	27	30	3
12	31	31	0	26	26	0
13	30	31	1	25	26	1
14	31	33	2	27	28	1
15	31	31	0	26	27	1
16	30	33	3	25	28	3

Table 2. *Cont.*

Zone of Inhibition (Diameter in Millimeters)						
Sample Number	**CTX-30 µg**	**CTX + CV (30 + 10) µg**	**Difference in Diameter**	**CAZ-30 µg**	**CAZ + CV (30 + 10) µg**	**Difference in Diameter**
17	30	32	2	27	28	1
18	33	34	1	29	30	1
19	27	28	1	24	26	2
20	28	30	2	25	25	0
21	31	32	1	27	28	1
22	30	31	1	26	26	0
23	28	29	1	25	26	1
24	28	28	0	25	26	1
25	28	30	2	24	27	3
26	29	30	1	27	27	0
27	28	29	1	30	30	0
28	30	30	0	29	29	0
29	29	29	0	27	28	1
30	30	30	0	26	27	1
31	28	30	2	26	26	0
32	32	34	2	31	31	0
33	32	32	0	28	29	1

3.2. Sanitary and Hygienic Practices in Retail Meat Shops

A survey of meat shop representatives revealed that 45.45% (45/99) of them had formal training in slaughtering practices (Table 3). In addition, 60.61% (60/99) of them wore protective clothing, and 30.30% (30/99) were aware of possible *E. coli* contamination in meat. However, only 15.15% (15/99) of the retail meat shop personnel used gloves to handle, chop, and sell meat. The chopping boards were mostly made of wooden materials. The majority of the representatives were aware of the importance of washing their hands, but only 5.05% (5/99) of the meat shops had separate equipment for cleaning the viscera of animals. Only 40.4% (40/99) of the shops had a proper drainage facility, while only 20.2% (20/99) of them had a continuous supply of clean water. Hence, they stored water in drums and buckets. Around 65.66% (65/99) of the meat shops had a cold storage facility to store meats (Table 3).

Table 3. Summary of the survey regarding the sanitary status and hygienic practices of retail meat shops.

Total No. of Meat Shops Surveyed = 99			
Serial Number	**Practices**	**Positive Observation/Response**	**%**
1	Formal training in meat processing	45	45.45
2	Use of protective/separate clothing	60	60.61
3	Knowledge of *E. coli* contamination of meat	30	30.30

Table 3. *Cont.*

Total No. of Meat Shops Surveyed = 99			
Serial Number	**Practices**	**Positive Observation/Response**	**%**
4	Hand sanitation before and after cutting meat	75	75.76
5	Use of gloves during meat handling	15	15.15
6	Concrete floor without cracks	70	70.71
7	Proper drainage	40	40.40
8	Regular cleaning of equipment	80	80.81
9	Continuous supply of clean water	20	20.20
10	Availability of deep freeze	65	65.66
11	Smooth and easily washable cutting board	50	50.51
12	Separate equipment for viscera	5	5.05

4. Discussion

E. coli bacteria are normally found as a commensal organism in the gastrointestinal tracts of animals and humans [6]. Some harmful strains of *E. coli*, such as Shiga-toxin-producing *E. coli*, cause abdominal cramps, bloody diarrhea leading to hemorrhagic colitis, and other severe complications such as hemolytic uremic syndrome [23,24]. These strains are mostly transmitted through the consumption of contaminated food, water, raw or uncooked meat, unpasteurized milk, contaminated equipment, unhygienic food handling practices, rodents, insects, etc. [25]. This study was performed to screen for *E. coli* in the meat dispatched to the consumers in Bharatpur Metropolitan City of Chitwan, Nepal, by collecting chopping board meat samples, and we observed that one third of the specimens were positive for *E. coli*.

Though *E. coli* were detected in 33.33% (33/99) of the chopping board meat samples, ESBL-producing *E. coli* were not detected in any of the tested samples from the meat shops. An earlier study in Nepal showed the frequency of the ESBL-producing strain of Gram-negative bacteria in chicken meat samples to be around 36.9% (38/103) [26]. In that study, meat samples were collected from the thighs, breasts, and wings of chicken from slaughterhouses, and various Gram-negative bacteria, including *E. coli*, were studied [26]. In our study, we collected leftover samples from chopping boards. We did not distinguish between chevon or poultry meat and did not look for any other bacteria except for *E. coli*. Several other studies within Nepal, both in animals and humans, have indicated the detection of ESBL-producing bacteria. For example, 4 of 27 *E. coli* isolates were reported to be ESBL producers in poultry fecal samples collected from commercial poultry farms in Kirtipur, Nepal [27]. Similarly, when 113 rectal swab specimens were collected and tested from backyard and commercial chicken in Kaski, Nepal, 30.1% (34/113) of the samples were ESBL-positive, and they were predominantly *E. coli* isolates [28]. In a subsequent study, the same group of researchers isolated ESBL-producing *Enterobacteriaceae* from rectal swab samples of buffalo, cows, and goats raised by a subsistence farming community in Kaski District [29]. They also isolated ESBL-producing bacteria from rectal swabs of children and adults of the same households [29]. When stool specimens were tested from apparently healthy students in Kathmandu, Nepal, ESBL-producing *Enterobacteriaceae* were identified in 9.8% of the specimens [30]. Similarly, among 514 Gram-negative bacilli urinary isolates collected from a tertiary care hospital in Lalitpur, Nepal, 6.8% (35/514) were ESBL producers, and among them 33 were *E. coli* [31]. When 109 sputum samples of patients with lower respiratory tract infections were analyzed in Bharatpur hospital, Nepal, 31 samples showed Gram-negative bacteria, 15 were multidrug-resistant, and 9 were ESBL producers [32]. These findings suggest the possibility of the transmission of ESBL-

producing bacteria at the human–animal interface and the need for regular studies to determine the existing situation of the AMR pattern in animals and humans in Nepal.

The survey results of the sanitary and hygienic practices in the retail meat shops indicated that they are lagging in maintaining the proper sanitary standards, which was further backed up by the presence of *E. coli* in one third of the tested samples. Poor sanitary and substandard hygienic conditions in meat shops were also reported in other areas of Nepal. A survey conducted on the hygienic practices in retail meat shops in Butwal municipality, Nepal, indicated that the retail meat shops had poor hygiene levels and were lacking basic facilities such as sewage disposal systems, drainage facilities, and hand washing basins [33]. A descriptive cross-sectional study in Dharan municipality, Nepal, also concluded that the hygienic practices were found to be unsatisfactory [34]. Another cross-sectional field-based study on Ratnanagar municipality, Nepal, also reported that 52.6% (60/114) of slaughterhouse personnel had a poor level of sanitary and hygienic practices [35]. Slaughterhouses should have a continuous supply of clean water; a proper drainage system; good electricity and a good transportation facility; and separate spaces for different animal procedures, including antemortem inspection, postmortem processing, and the dressing and packaging of meats [36,37]. Proper refrigeration facilities should be available at slaughterhouses, as they enhance the shelf life of meat and reduce the growth of microorganisms. Our findings and the observation from other studies suggest the need for the strict implementation of the 'Animal Slaughterhouse and Meat Inspection Act 1999' [38] in Nepal, which aims to safeguard the health of the consumer through the production of safe and hygienic meat.

The retail meat shop personnel had minimal training, and only 30.3% of them were aware of *E. coli*. An earlier study explored the factors associated with meat safety knowledge and practices among slaughterhouse personnel in Chitwan, Nepal, and showed that they had neither adequate knowledge on meat safety nor good slaughterhouse practices [35]. Having secondary and higher-level education and butchering as a sole occupation were among the factors associated with more knowledge on meat safety [35]. We observed that only 5.05% of the retail meat shops had separate equipment for viscera and only 15.15% of the meat shop personnel used gloves. An earlier study carried out among pork handlers and within pig meat shops in Chitwan, Nepal, in association with campylobacteriosis risk factors also had similar findings [39]. They reported the absence of dirty sections in the meat shops and a lack of glove wearing [39]. These findings indicate that meat shops in Chitwan district, irrespective of the types of meats they sell, have poor sanitary and hygienic conditions, and there is need for improvement. This can be achieved by providing training to the slaughterhouse and meat shop personnel and by creating awareness of meat safety and hygienic practices.

5. Conclusions

One third of the chopping board meat samples collected in Bharatpur Metropolitan City were positive for *E. coli*. However, none of the samples were positive for ESBL-producing *E. coli*. The hygienic and sanitary conditions of the retail meat shops were of inferior quality. While the continuous surveillance of pathogenic microorganisms is necessary in dispatched meat samples, there is a need to improve the quality of slaughterhouses and retail meat shops.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

The questionnaire used to interview representatives of retail meat shops in Bharatpur Metropolitan City, Nepal, and during the inspection of meat shops to understand the sanitary and hygienic practices.

I . . . . . (Name of Participant) am willingly answering the questions being asked to be used for research purpose and provide my consent to use the information without violating my privacy.
Participant
(Signature)

Participant Details:

1. Name of Slaughter House: . . . .
2. Address: . . . . . .
3. Qualification & formal training on meat handling/selling:
   - ☐ Yes
   - ☐ No

Slaughterhouse management:

1. Slaughter species: . . . . . .
2. Average sales per day: . . . . . .
3. Knowledge about *E. coli* poisoning/contamination of meat?
   - ☐ Yes
   - ☐ No
4. Do you wash hands before and after cutting meat?
   - ☐ Yes (before and after)
   - ☐ Yes (only after)
   - ☐ No
5. Floor type
   - ☐ Concrete (Cement/Tile without cracks)
   - ☐ Mud
6. Regular clean water supply.
   - ☐ Yes
   - ☐ No

If yes, what is the source?.....

7. Cleanliness
   - ☐ Clean
   - ☐ Moderately clean
   - ☐ Dirty
8. Deep freeze availability . . . . . .
   - ☐ Present
   - ☐ Absent
9. House management
   - ☐ Open (no covering in the chopping area)
   - ☐ Closed (proper covering in and surrounding chopping area by appropriate materials)

10. Nature of chop board
    - ☐ Smooth and easily washable
    - ☐ Difficult to clean properly
11. Chop board cleanliness
    - ☐ Clean (flaming and scrapping with a knife before or after chopping)
    - ☐ Moderately clean (scrapping only before or after the chopping)
    - ☐ Dirty (no such activity performed)
12. Separate equipment available for viscera.....
    - ☐ Yes
    - ☐ No
13. Use of gloves during meat handling.....
    - ☐ Yes
    - ☐ No
14. Regular cleaning of equipment.....
    - ☐ Yes
    - ☐ No
15. Proper drainage facility . . . .
    - ☐ Present (proper drainage, continuous flow of wastewater in the drainage)
    - ☐ Absent (stagnant wastewater)
16. Use of separate/protective clothing (e.g., apron) during meat handling . . . . . .
    - ☐ Yes
    - ☐ No

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