# Antibiotic Resistant Profile of Coliform and *Staphylococcus aureus* Isolated from Paneer Sample of Kathmandu Valley

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**Abstract**

Microbial contamination with antibiotic- resistant bacteria are major threats to public health in Nepal. This study aims to detect microbial quality of paneer and determine antibiotic susceptibility pattern of coliforms and *S. aureus* isolated from Paneer. For this, 30 paneer samples were randomly collected from dairy shops in Kathmandu and respective bacterial count were determined by using pour plate technique. All isolated organisms were identified by biochemical tests using appropriate selective media. Antibiotic Susceptibility Testing (AST) was carried out by Kirby Bauer disk diffusion method. In this study, Total Bacterial Count (TBC) of 18 (60.1%) samples, Total Coliform Count (TCC) of 5 (16.6%) samples and Total Staphylococcal Count (TSC) of 6 (50%) samples were within the limit of FSSAI guideline (2012). A total of forty-six bacteria were isolated in this study, of which 34 (73.9%) were identified as coliforms and 12 (26.1%) were identified as *S. aureus.* The coliform bacteria showed higher resistance towards Azithromycin (100%) followed by Cefoxitin, Ampicillin, Amikacin, Levofloxacin, Nalidixic acid, Chloramphenicol, Tetracycline and Gentamicin in descending order. While in case of *S. aureus,* higher resistance was shown against Penicillin. G (41.7%) and Nalidixic acid (41.7%) followed by Cefoxitin, Ampicillin, Azithromycin, Chloramphenicol and Levofloxacin. 4 (33.4%) MRSA were identified based on their full resistance towards Cefoxitin. Also, 24 multiple-drug resistance (MDR) bacteria were detected and higher MDR was observed among coliforms 21 (87.5%). To summarize, the maximum number of samples exceeding microbial quality specification and presence of multiple antibiotic resistance among isolated bacteria signifies poor quality of the paneer available in the market and can be considered a possible threat to consumers. Therefore, it is utmost necessary to monitor and control the quality of paneer sold in Kathmandu.

## 1. Introduction

Paneer is a non-fermented cheese made from milk, and can become microbiologically hazardous because of contamination directly from contaminated milk or poor hygiene during preparation (Deshmukh and Vyas, 2016). Such conditions may cause different foodborne diseases

to the consumer. It is evident that about 7.69% individuals of world populations suffer from foodborne diseases every year and 7.5% of all deaths annually are due to foodborne illness (WHO, 2015). The contamination of paneer can cause gastrointestinal illness in the consumers. Foodborne diseases, especially infectious dairy products, are not limited to third world countries.

Among many microorganisms, *Escherichia coli* and *Staphylococcus aureus* are the most frequent contaminants in food products. Even the Staphylococcal enterotoxin outbreak has always been an indispensable threat to dairies (Abril *et al*., 2020). Further, food contamination with antibiotic resistant bacteria can be a serious risk to public health (Hassani *et al*., 2022; Arbab et al., 2021), as the antibiotic resistant trait can be transferred to other pathogenic bacteria of human clinical relevance (CDC, 2019). Antibiotic resistance is a worldwide problem (Liu *et al*., 2015) and available studies around the world have affirmed about the occurrence of multidrug resistant *E. coli*, ESBL toxin producing. The Centers for Disease Control and prevention (CDC) estimates that each year more than 2 million persons are infected with antibiotic resistant microbes in US and at least 23.000 people die due to these infections (Akova M., 2016). The global occurrence of antimicrobial resistance in *E. coli* over the years is worrying and highlights the necessity for appropriate interventions to prevent transmission. Nepal is considered a major contributor to the growing burden of antimicrobial resistance (Kakchapati *et al*., 2021). Moreover, MRSA facilitated by mecA gene, is one of the prime problems around the world including Nepal. And this MRSA has been reported to be 15-69% from different areas in the Nepalese setting (Khanal et al., 2018). Therefore, implementation of the appropriate hygienic controlling unit for milk and milk products throughout the food production chain is crucial to ensure safety. Additionally, regular surveillance of the microbial quality of milk and milk products along with antibiotic resistant profile trend of milk borne pathogens is a necessity to ensure safe guard for the consumers.  So, this study provides an insight of the microbial quality and antimicrobial resistance (AMR) profile of bacteria isolated from the paneer available at the local vendors in Kathmandu valley.

## 2. Materials and methods

A total of 30 paneer samples from a local vendor were collected s in this study from different localities of Kathmandu (Naikap, Kalanki, Bafal, Sitapaila, Thamel). Random sampling technique was employed and sampling location was identified based on high population density. 6 samples from each location were collected in pre-sterile foils and packed carefully. The packed samples were transported to the laboratory within 2 hours of collection and processed within 30 minutes (USFDA, 2001).

## 2.1 Enumeration of bacteria

The collected paneer samples were weighed and suspended in the diluent. The suspended samples were serially diluted up to $10^6$ and plated in respective media using pour plate technique (Wehr and Frank, 2004). Plate count Agar (PCA) was used for TBC determination and incubated at $37^\circ$C. Violet red bile agar (VRBA) was used for TCC and TFC using double layering techniques

and incubated at $37^\circ$C and $44^\circ$C for enumeration of coliform and thermotolerant coliform respectively. However, the diluted samples were plated by spread plate technique on Mannitol salt agar (MSA) for TSC determination and incubated at $37^\circ$C (ISO, 2012).

## 2.3 Isolation and identification of bacterial isolate

Identical pink color colonies from VRBA plates and straw yellow colonies from MSA plates were sub cultured separately and tested for their biochemical characteristics. The isolates were identified and confirmed by exploring a series of biochemical tests that are specific to Gram-negative and Gram-positive bacteria. Biochemical tests such as IMVIC were used for identifying coliform bacteria, while the coagulase test was used to identify *Staphylococcus aureus* (Cheesbrough M., 2016).

## 2.4 Antibiotic susceptibility testing

All isolates were proceeded to antibiotics susceptibility testing using Kirby Bauer disk diffusion method on Muller Hinton agar (MHA) according to CLSI guidelines (2018). The antimicrobial agents tested for *Staphylococcus spp* were Levofloxacin (5mcg), Tetracycline (30mcg), Cotrimoxazole (25mcg), Cefoxitin (30mcg), Ampicillin (10mcg), Chloramphenicol (30mcg), Ampicillin (10mcg), Penicillin G (10mcg), Gentamicin (10mcg) and Nalidixic acid (30mcg). While for coliform, the antibiotics used were Ampicillin (10mcg), Chloramphenicol (30mcg), Nalidixic acid (30mcg), Cotrimoxazole (25 mcg), Tetracycline (30mcg), Cefoxitin (30mcg), Levofloxacin (5mcg), Amikacin (30mcg), Azithromycin (15mcg) and Gentamicin (10 mcg). Zone of inhibition diameter was measured and the results were interpreted based on CLSI interpretation criteria (WHONET 2020). All intermediate isolates were placed in the resistant category. Data analysis was done using SPSS (Version 20) and WHONET 2020 software.

## 3. Results and discussion

### 3.1 Bacterial count

A total of 30 paneer samples were processed for microbial count determination. The average bacterial count was $1\times10^6$ CFU/gm, and 18 (60.1%) samples had TBC that was within the acceptable limit of the FSSAI guideline (2012). This was lower than the study carried out by Godbole *et al.* (2013). In the case of TCC, the average count was $6\times10^4$ CFU/gm in this study which was higher count as compared with Dhole *et al.* (2009). A total 5 (16.6%) samples had TCC within the acceptable limits of DFTQC as well as FSSAI. This higher number of samples (83.4%) exceeding the acceptable TCC limit was lower than the study reported by Desale *et al.* (2009). While the average thermotolerant coliform count was $7.7\times10^3$ CFU/gm which was higher from the study reported by Poudel and Sah (2015). Such higher coliform load may be due to contamination occurring from different sources including bad quality of raw milk, transportation, manufacturing, handling, storage, packaging in uncontrolled environments that may even lead to diarrheal diseases, gastroenteritis, food poisoning. Coliforms are indicators of fecal contamination, therefore the source of contamination for their presence in paneer may be via the environment.

However, for TSC, six (50%) samples were found to be within the acceptable limit of the FSSAI guideline (2012). Also, the average TSC count was lower than the study performed by Godbole *et al.* (2013). The high Staphylococcal count may be due to the fact that *Staphylococcus* is a normal flora and it is omnipresent in the settings of farms and people. It may have found its way into paneer via contaminated milk or poor hygienic conditions in their manufacturing and packaging.

## 3.2 Isolation and identification of paneer isolates

A total of 46 bacteria were isolated and identified.  Of total isolates, 34(73.9%) were identified as coliforms and 12(26.1%) were identified as *S. aureus.* Among 34 coliforms, 15(44.1%) were *E. coli*, 14(41.2%) were *Klebsiella* spp. and 5(14.7%) were *Citrobacter* spp. The identification of the total bacterial isolates revealed that coliforms were higher in number. And among coliforms, the predominant was *E. coli* followed by *Klebsiella* spp., *S. aureus* and *Citrobacter* spp. This was similar to the findings of Gogoi and Sultana (2018), NCRP (2019) and Amosun *et al.* (2017), who reported higher prevalence of *E. coli.* But in the case of *S. aureus,* Amosun *et al.* (2017) reported a lower percentage than our study. The predominance of coliforms in our paneer samples is not a good sign as coliforms are considered as an indicator organism for contamination (especially fecal origin Thermotolerant *E. coli*).  These coliforms may have accesses into the paneer sample either during the processing or directly through the contaminated milk.

## 3.3 Antibiotic susceptibility pattern

All 46 isolates were tested for their susceptibility towards different antimicrobials. Out of those, 34 coliforms showed 100% susceptibility towards Cotrimoxazole followed by Gentamycin (97%), Chloramphenicol (94.1%), Tetracycline (94.1%), Nalidixic acid (82.3%), Levofloxacin (73.5%), Amikacin (64.7%), Ampicillin (35.2%) and Cefoxitin (32.3%). The AST plates of *E. coli* is shown in Figure 1. Additionally, the species wise susceptibility of coliforms is given in Figure 2.
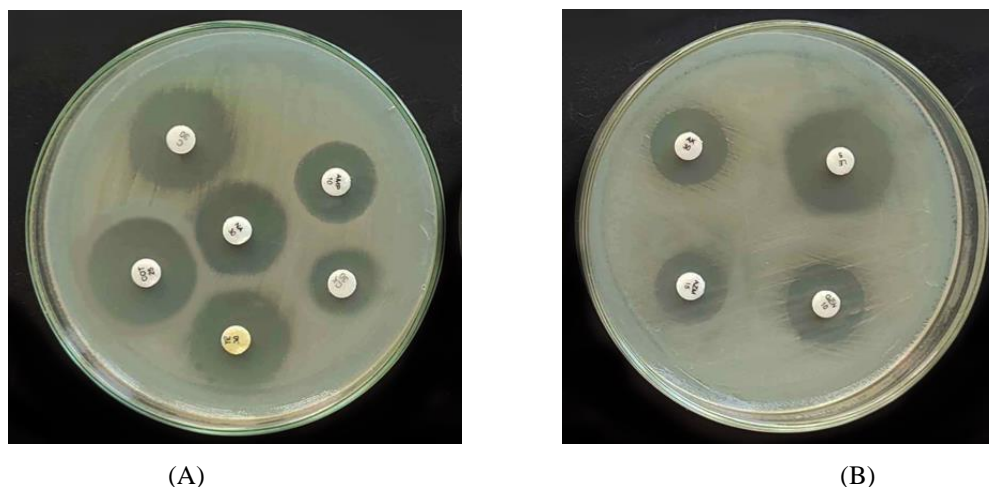


(A)                                                                (B)

**Fig. 1: Zone of inhibition shown against different antimicrobials by *E. coli.* (Plate A:  COT =Cotrimoxazole, TE = Tetracycline, CX = Cefoxitin, C= Chloramphenicol, A=Ampicillin, NA=Nalidixic Acid) (Plate B: AK=Amikacin, LE= Levofloxacin, AZM=Azithromycin, GEN=Gentamycin)**
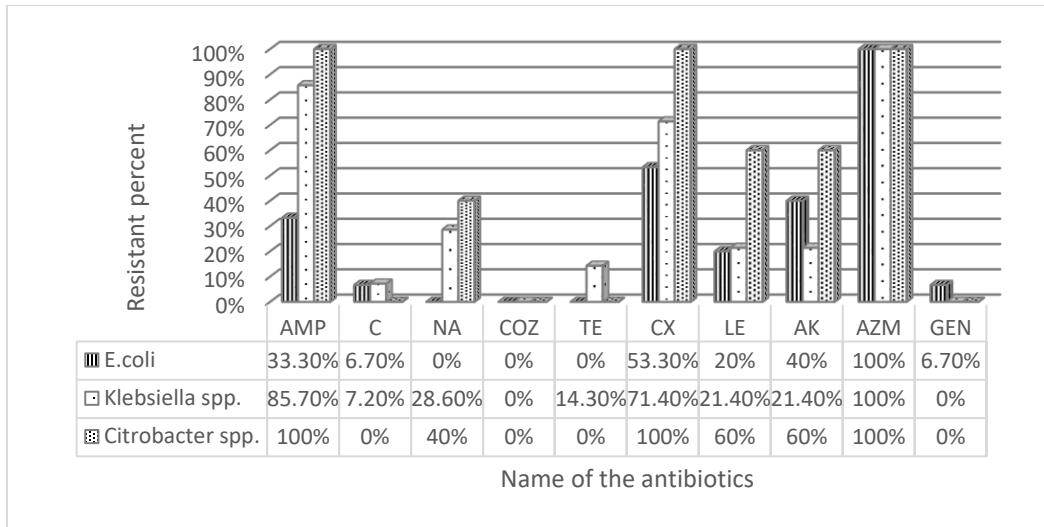
| Name of the antibiotics | AMP | C | NA | COZ | TE | CX | LE | AK | AZM | GEN |
|---|---|---|---|---|---|---|---|---|---|---|
| E.coli | 33.30% | 6.70% | 0% | 0% | 0% | 53.30% | 20% | 40% | 100% | 6.70% |
| Klebsiella spp. | 85.70% | 7.20% | 28.60% | 0% | 14.30% | 71.40% | 21.40% | 21.40% | 100% | 0% |
| Citrobacter spp. | 100% | 0% | 40% | 0% | 0% | 100% | 60% | 60% | 100% | 0% |

**Fig. 2: Percentwise distribution of antibiotic resistance in different species of Coliforms**

Among the coliforms, *E. coli* showed higher resistance towards Azithromycin which was similar to the finding of Phattepuri *et al.* (2020). Also, higher resistance was detected towards Ampicillin and Cefoxitin which supports the finding of Gogoi and Sultana (2018). Further, full susceptibility was shown by Tetracycline which seems contradicting with the finding of Gautam, *et al.* (2015). Chloramphenicol resistance (11.1%) was lower but was comparable to the finding of Amosun *et al.* (2017). Likewise, in the case of *Klebsiella* spp., higher resistance was observed towards Azithromycin followed by Ampicillin and Cefoxitin. However, lower resistance was observed towards Chloramphenicol which was contrasting to the finding of Amosun *et al.* (2017). Further, as for *Citrobacter* spp. full resistance was observed towards Ampicillin, Azithromycin and Cefoxitin. Additionally, higher resistance was observed towards Levofloxacin and Amikacin. According to Karczmarczyk *et al.* (2011), the development and spreading of these antibiotic resistance genes generally occurs via horizontal gene transfer, which is aided by many mobile genetic elements such as plasmids, transposons, and integrons.

In case of *S. aureus,* they also showed some important resistance that needed immediate attention. Among the twelve *S. aureus,* all the isolates were 100% susceptible towards Gentamicin, Cotrimoxazole and Tetracycline whereas resistance were observed towards Ampicillin (16.6%), Azithromycin (16.6%), Chloramphenicol (8.3%), Levofloxacin (8.3%), Nalidixic acid (41.7%) and Penicillin G (41.7%). The AST plates of *S. aureus* is shown in Figure 3. However, in case of Cefoxitin, 4(33.4%) isolates showed full resistance towards Cefoxitin were identified as Methicillin-resistant *Staphylococcus aureus* (MRSA). *S. aureus* showed higher resistance towards Penicillin followed by Cefoxitin which is similar to the finding of Matallah *et al.* (2019) and Gogoi *et al.* (2018). Cefoxitin resistance is an indicator of MRSA (Smyth and Kahlmeter, 2005) and such prevalence of MRSA in Paneer sample has been continuously reported in various studies (Joshi et al., 2014; Haren et al., 2012). In the diagnostic laboratories, the MRSA strains are confirmed by

detecting of mecA gene (Elimam et al., 2014) and Cefoxitin is a powerful inducer of the mecA regulatory system. Thus, it is generally recommended by different studies to use Cefoxitin for the detection of MRSA when using disk diffusion testing (Anand et al., 2009).
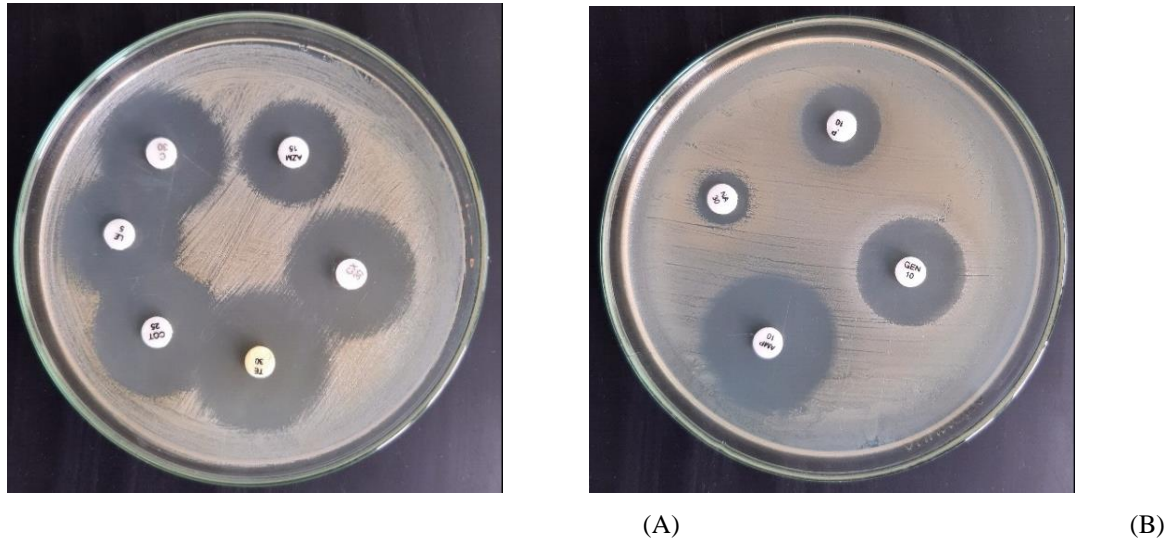


(A)                                                                      (B)

**Fig. 3: Zone of inhibition shown against different antimicrobials by *S. aureus*. (Plate A:  COT =Cotrimoxazole, TE = Tetracycline, CX = Cefoxitin, C= Chloramphenicol, AZM=Azithromycin, LE= Levofloxacin) (Plate B:  A=Ampicillin, NA=Nalidixic Acid, Penicillin G, GEN=Gentamycin)**

Besides MRSA, multiple resistance towards 3 or more classes of antibiotic was also observed among the isolates. Among the total 46 isolates, 24 were identified as multiple-drug resistance (MDR). Of the 24 MDR, 6(25%) were *E. coli*, 10(41.7%) were *Klebsiella* spp., 5(20.8%) were *Citrobacter* spp. and 3(12.5%) were *S. aureus*. Out of total, over half of the isolates were identified as MDR among which   *Klebsiella* spp. showed higher counts followed by *E. coli* and *Citrobacter* spp (Figure 2). The distribution of MDR across the coliforms and *S. aureus* was not statistically significant (p= 0.092). Such prevalence of antibiotic resistance in bacteria from paneer samples is surely a microbial contamination either through milk contamination (Hassani et al., 2022) or through environmental sources during production, packaging or handling. If the contamination in paneer is from milk samples then the high percent of MDR may be due to high and unchecked usage of antibiotics in cattle for breeding and treatment like the use of beta-lactam antibiotics to treat mastitis in cattle. Evidently, higher proportion of MDR in milk has been reported by Rai *et al.* (2020) in Kathmandu. Further, if the contamination is due to the environment or contact surface, then it may be due to a gene transfer or mutation in the bacteria. Whatever the source of contamination, the presence of multiple antibiotic-resistant bacteria in paneer or any milk product is a serious issue. And the study of Karczmarczyk *et al.* (2011) even highlighted that the emergence and circulation of antimicrobial resistance in the food chain is an important public

health concern. Therefore, routine surveillance of the AMR profile of food isolates is necessary to ensure the safety of consumers.

## 4. Conclusion

Microbial contamination in paneer is evident and antimicrobial resistance of those microbial towards common antimicrobials seems to be emerging. Therefore, good hygienic practices from production to market level are required to reduce the antibiotic resistant bacteria load in paneer available in Kathmandu valley.

## Authors' contribution

MP and VK contributed in designing, conducting, data analysis and writing; AKS contributed in designing and supervising; ST contributed in planning, supervising, data analysis and writing of the manuscript.

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