ANTIMICROBIAL RESISTANCE AND MOLECULAR CHARACTERIZATION OF *ESCHERICHIA COLI* ISOLATED FROM MILK AND MILK PRODUCTS IN CHHATTISGARH

M.V.Sc. Thesis

By

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COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY

DAU SHRI VASUDEV CHANDRAKAR KAMDHENU VISHWAVIDYALAYA, DURG

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VITA

The author of this thesis, Kavita Raj was born on february 16th, 1996 at Amandula, Janjgir-Champa (C.G.). She passed her higher secondary examination in the year 2013 with grade one from Vivekanand Higher Secondarary School, Mana Camp, Distt. Raipur (C.G). In July 2015 she started her career in veterinary profession for her B.V. Sc. and A.H. from College of Veterinary and A.H, Anjora, Durg (C.G.) and completed it in July 2020 with OGPA of 6.35 /10.00. Due to her keen interest in research work towards public health, she joined the Department of Veterinary Public Health and Epidemiology in College of Veterinary Science and Animal Husbandry, Anjora, (C.G) for her Master's degree in August 2020. She has completed the requisite course works for M.V.Sc. program.

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CERTIFICATE-I

This is to certify that the thesis entitled **"Antimicrobial resistance and Molecular Characterization of *Escherichia coli* isolated from milk and milk products in Chhattisgarh"** submitted in partial fulfilment of the requirements for the degree of **"Master of Veterinary Science"** of Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Durg, is a record of bonafide research work carried out by **Kavita Raj** under my guidance and supervision. The subject of the thesis has been approved by Student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma (certificate awarded etc.) or has been published/published part has been fully acknowledged. All the assistance and help received during the course of investigations have been duly acknowledged by her.

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Member: Dr. Tripti Jain **……...**

CERTIFICATE-II

This is to certify that the thesis entitled **"Antimicrobial resistance and Molecular Characterization of *Escherichia coli* isolated from milk and milk products in Chhattisgarh"** submitted by **Kavita Raj** to the Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Durg, in partial fulfilment of the requirements for the degree of **"M.V.Sc."** in the Department of Veterinary Public Health and Epidemiology, College of Veterinary Science and Animal Husbandry, Anjora, Durg, has been approved by the Student's Advisory Committee after oral examination in collaboration with the external examiner.

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Though every possible effort was made to bring out a complete and comprehensive thesis on the selected topic with the able guidance of my supervisor, errors, if any, are purely mechanical and solely mine.

Date:

Anjora, Durg ***Dr. Kavita Raj***

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ABBREVIATIONS

Abbreviations	Full form
%	Percent
°C	Degree Celcius
µl	Microlitre
bp	Base pair
CA	Clavulanic acid
CDC	Centre for disease control and prevention
CLSI	Clinical laboratory standard institute
CTX-M	Cefotaximase
DDM	Disc diffusion method
DNA	Deoxyribonucleic acid
EFSA	European food safety authority
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMB	Eosine methylene blue agar
EPEC	Enteropathogenic *E. coli*
ESBL	Extended-spectrum beta-lactamase
et al.	Et alia (and others)
ETEC	Enterotoxigenic *E. coli*
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
Fig.	Figure
Gm	Gram
GPPW	Glucose phosphate peptone water
gyrA	DNA gyrase subunit A
Hrs	Hours
i.e.	id est (that is)
ICMSF	International Committee for microbiological Specification for foods
IMViC	Indole, Methyl Red, Voges-Proskauer, Citrate
MAR	Multiple antibiotics resistance
mcg	Microgram
MDR	Multi-Drug Resistant
mg	Milligram
MHA	Mueller Hinton agar
min	Minute (s)
ml	Millilitre
MLA	MacConkey's Lactose Agar
mm	Milli metre
NA	Nutrient agar

ParC	DNA topoisomerase 4 subunit A
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
rpm	Revolution per minute
SHV	Sulphydryl variable
TBE	Tris-Borate EDTA
TEM	Temoneria
tet	Tetracycline resistance protein
V	Volts
viz.	Vide licet (namely)
WHO	World Health Organisation
α	Alpha

CHAPTER-I

INTRODUCTION

Food-borne diseases leads to around 600 million illnesses, 4,20,000 deaths and 33 million disability-adjusted life years (DALYs) every year throughout the world (Kumar *et al.,* 2021). In India, foodborne disease causes approximately 100 million illnesses, 1,20,000 deaths and 8 million DALYs are lost each year. According to Integrated Disease Surveillance Project (IDSP) report, nearly 40% of all outbreaks reported in India between 2011 and 2017 were foodborne and leads to acute diarrheal diseases (Kumar *et al.,* 2021). Up to 70% of diarrheal diseases reported in developing nations like India are thought to be due to eating contaminated food (WHO, 2008).

Milk is a major part of human food and plays a prominent role in the human diet. It is regarded as the most complete food found in nature. Milk contains protein and calcium and is a good source of vitamin B_{12}, thiamine and riboflavin. Its high nutritional value provides the ideal environment for bacteria to quickly multiply, particularly when produced and stored at room temperature under unhygienic conditions. Microorganisms in raw milk can come from a variety of places, including the animal, air, milking equipment, feed, soil, grasses (Badri *et al*., 2017). In India peoples traditionally consumes raw milk at small farms where it is produced or processed into a variety of foods. Handling the milk under unhygienic conditions is matter of concern in India. Therefore, the risk of contamination of milk and milk products could be much higher when the milk is processed at the household level (Gran *et al.,* 2003)

According to Centre for Disease Control and Prevention (CDC) from 1993 through 2012, 127 outbreaks reported were linked to raw milk. Consumption of unpasteurized milk increases the risk of foodborne illness in consumers by 150 times (Bajrami and Sulaj, 2017). *Escherichia coli* is a significant cause of bacterial diarrhoea in humans, among the numerous enteric bacterial infections (Lanjewar *et al.*, 2010). *E. coli* was frequently found in animal intestines. *E. coli* was frequently observed to infect cow's mammary glands during parturition and the first few weeks of lactation, which could result in acute and localized mastitis (Liu *et al*., 2021). *E. coli* is a gram-negative, non-spore-forming coccobacillus and is a member of the *Enterobacteriaceae* family. Its pathogenic forms cause gastrointestinal and extra-gastrointestinal diseases, such as urinary tract infections, meningitis, peritonitis and septicaemia (Tadesse *et al*., 2012).

In recent years, the prevalence of antimicrobial resistance (AMR) among microorganisms isolated from animal source foods has increased. Antibiotics are regularly added to feed as feed additives in intensive animal production at sub-therapeutic doses to stimulate growth, improve feed efficiency and avoid infections. The extensive use of antibiotics in human treatment, animal therapy and agricultural use as growth promoters are further causes of bacterial resistance to antibiotics (Vaishali, 2019). AMR is caused by a number of factors, including a delay in starting effective treatment, inadequate final therapy and increased aggressiveness of some resistant bacteria (CLSI, 2009). Approximately 65% of the global market for antibiotics is made up of β-lactam antibiotics (Thakuria and Lahon, 2013). *E. coli* is associated with the potential occurrence of antibiotic-resistant bacteria and their quick development (Ntuli *et al.,* 2016). Bacteria may

develop antibiotic resistance as a result of improper antibiotic choice and abuse (Da Silva and Mendonça, 2012). According to FAO, AMR is thought to be responsible for about 5,00,000 human deaths annually, worldwide. By 2050, the threat posed by AMR is expected to increase and resulting in an estimated 10 million deaths annually (Gundran *et al.*, 2019).

A rising global public health issue is extended-spectrum beta-lactamase (ESBL) *E. coli* and sources of clinically significant ESBL bacteria are continually emerging in the agri-food sector. The production of ESBL by *E. coli* is constantly posing a threat to global public health. The remarkable ability of ESBL, a plasmid-mediated lactamase enzyme, to hydrolyze penicillin, third- and fourth-generation cephalosporins, monobactams with the exception of carbapenem and cephamycin, has earned it worldwide recognition. These enzymes were mainly produced by bla_{TEM} and bla_{SHV} genes due to the burden on environmental health. $bla_{\text{CTX-M}}$, a new family of ESBL genes, recently gained popularity on a global scale (Kamaruzzaman *et al*., 2020). The development of beta-lactamases demonstrates there are risks to human health, such as the potential for resistance genes to be horizontally transferred (Skockova *et al*., 2014).

Due to their development of antibiotic resistance, gram-negative bacteria constitute a therapeutic challenge not only in medical settings but also in the general public. ESBL, AmpC beta-lactamase, efflux mechanism and porin loss are a few ways gram-negative bacteria develop drug resistance (Martinez *et al.*, 1999). There are various reports from North America, South America, Europe, Africa and Asia about the prevalence of the bacteria that produce ESBLs (Thakuria and Lahon, 2013). These ESBL-producing bacteria have become

significantly more common in recent years (Itokazu *et al.*, 1996). These enzymes pose a severe threat of widespread transmission across the gram-negative community and are typically found in integrons on plasmids (Doumith *et al.*, 2009). Variable susceptibility rates exist for fluoroquinolones, aminoglycosides and fourth-generation cephalosporins in the organisms that produce ESBL (Lautenbach *et al.*, 2001; Kariuki *et al*., 2007). By using beta-lactamase inhibitors like clavulanic acid (CLA) can be reduced the activity of ESBL. Antibiotic resistance increases illness costs, morbidity and death, especially for infections brought on by ESBL-producing bacteria (Tekiner *et al*., 2016). By 2030, antibiotic uses in animals in the BRICS (Brazil, Russia, India, China and South Africa) nations are predicted to be double in the region due to intensifying animal production and rising demand due to a growing population (Jindal *et al.*, 2021).

The high presence of bacteria that produce ESBLs and are multidrug-resistant in raw milk poses a possible health concern to anyone who consumes raw milk and milk products (Karuppasamy *et al*., 2014). The current investigation aims to evaluate the presence of multidrug resistance (MDR) *E. coli* in milk and milk products.

OBJECTIVES

1. **To isolate and identify *E. coli* from milk and milk products**
2. **To identify multidrug resistant isolates of *E. coli***
3. **Molecular confirmation of ESBL genes in drug resistant isolates**

CHAPTER-II

REVIEW OF LITERATURE

The presence of bacteria in food causes unpleasant changes in flavor, aroma, color and texture. One of the most dangerous foodborne pathogens, *E. coli* greatly affects food quality and safety. *E. coli* variants cause septicaemia, gastroenteritis and urinary tract infections. Bacterial resistance to antibiotics can be transmitted to humans through food-borne channels, water contamination, other environmental pathways, direct animal contact. Antibiotic-resistant bacterial infections may involve using second line antibiotics for treatment. Additionally, the commensal bacterial microflora may operate as a repository for resistance genes that can be shared by other bacterial species, including those that can infect both people and animals (EFSA, 2008).

It is required to pay attention to the potential of milk as a source of zoonotic bacterial diseases transmission that could manifest and harm the people. Zoonotic bacterial infections can be spread from animals to people in several ways, including through tainted animal products like milk and milk products, direct fecal-oral route, improper food handling and cooking. According to one health concept, humans close to animals can contract zoonotic pathogenic bacteria that can spread to other humans in the community (Widodo *et. al.,* 2022).

2.1. Genus *Escherichia coli*

In 1885 Theodor Escherich first identified *E. coli* from new born faeces which belongs to family *Enterobacteriaceae* and given the scientific name *Bacterium coli commune* (Croxen *et al.,* 2013). It is a facultative anaerobic bacterium that is gram-negative, non-spore forming, flagellated, rod-shaped and

about 1 µm in diameter. *E. coli* is the one of the most significant global contributors to foodborne illness. *E. coli* strains are passed from animal to human is through milk and dairy products is one of the primary route of footborne illness (Dehkordi *et al.,* 2014)

Pathogenic *E. coli* have been divided into various pathotypes that causes common diseases. The pathogenic strains of *E. coli* are enterotoxigenic *E. coli* (ETEC) which causes diarrhoea, enteropathogenic *E. coli* (EPEC) which causes traveler's diarrhoea, enterohaemorrhagic *E. coli* (EHEC) which causes bloody diarrhoea, enteroinvasive *E. coli* (EIEC) which causes dysentery-like diarrhoea (Nataro and Kaper, 1998).

2.2. Isolation and Identification of *E. coli*

E. coli is a facultative anaerobe that may be easily isolated from food in the general or specific medium at 37° C under an aerobic environment. They also found that the organism can grow at 44° C (Edwards and Ewing 1972).

E. coli can be isolated from food using general or specific media at 37˚C in an aerobic environment. To differentiate enteric organisms based on morphology. Members of the *Enterobacteriaceae* family were selectively grown on MacConkey or EMB agar (Balows *et. al.*,1991).

Indole test was the single best test for differentiating *E. coli* strains from other *Enterobacteriaceae* members, which found positive in 99 percent of *E. coli* strains (Kaper and Nataro, 1998).

E. coli strains were frequently isolated from MacConkey agar plates and produce lactose-fermenting pink colonies. They observed, only 90% of *E. coli* isolates were lactose positive (Croxen *et al*., 2013).

2.3. Biochemical characterization of *E. coli*

Atypical biochemical behaviour of *E. coli* strains (citrate positive) has been reported by (Dubey and Sharda, 2001).

Choudhary, (2012) conducted several biochemical test for preliminary identification of *E. coli* strains. Almost all *E. coli* isolates produced acid from glucose, lactose, mannitol and arabinose but not from adonitol and inositol, whereas acid produced from dulcitol, salicin, and sucrose were found varies in different strains. All *E. coli* isolates were catalase, indole and methyl red positive, oxidase and Voges Proskeur negative and inability to grow on Simmon's citrate medium.

2.4. Prevalence of *E. coli* from milk and milk products

Kulshrestha *et al.,* (1990) collected samples from Bareilly, India and analyzed 14 samples of Dahi were isolated 10 *E. coli* whereas, from 3 Kalakand samples he revealed only 1 *E. coli* isolate.

Kumar and Prasad (2010) assessed the *E. coli* contamination in 135 raw milk samples and milk products from dairy farms, vendors, and homes around in Pantnagar, India. Out of all the milk samples, it was discovered that milk obtained from vendors had the most contamination (26%), followed by milk from dairy farms (20%), and milk from homes (6.6%). This finding may be related to the handling of milk in an unclean manner. The milk-related products with the highest levels of contamination were Burfi (33.3%), Dahi (20%), Gulabjamun (20%), Butter (20%), Khoa (13.3%) and Ice cream (6.6%).

Gundogan and Avci (2013) studied to determine the prevalence of 46.66% of *E. coli* isolates in raw milk samples collected from various supermarkets, dairy farms and pastry shops in Turkey. Out of 15 samples, 7 of these were found positive.

Rashid *et al.* (2013) carried out a study to determine the milk's hygienic status in Jammu and Kashmir, India. The presence of *E. coli* was found in 33.96% (18/53) of the 53 milk samples that were collected in the Jammu region and processed by standard procedure.

Virpari *et al.* (2013) collected 50 milk samples under aseptic conditions from milk vendors and retail stores in Anand, Gujrat, India. Further analysis of the samples showed that 52% of the raw milk samples had *E. coli*.

Lubote *et al.* (2014) analyzed 75 raw milk samples collected from small-scale dairy farmers, street sellers and outlet stores in Tanzania and 68 samples were tested positive for *E. coli* with prevalence of 90.67%.

Su *et al.* (2014) analyzed 412 raw milk from Southern Taiwan and reported a prevalence of (13.8%) isolates and 57 *E. coli* milk samples tested positive.

Yadav *et al.* (2014) evaluated the 25 raw milk samples and 25 pasteurized milk samples from different dairy shops in Allahabad city, India. The study found 68% of pathogenic bacteria isolates from pasteurized milk and 88% of pathogenic bacteria isolates from raw milk to be positive for *E. coli* incidence was found to significantly in 43.58% of cases.

Bhoomika *et al.* (2015) determines the prevalence of *E. coli* 81.11% (73/90) of raw milk found to be positive in Chhattisgarh.

Gautam *et al.* (2015) reported that the prevalence of *E. coli* was 22 (32.93%) in 75 raw milk samples processed from various locations in Allahabad, India. Isolated microorganisms were identified and confirmed based on culturally relevant physical and biochemical traits.

Tabaran *et al.* (2016) studied the incidence of enterotoxigenic *E. coli* (ETEC) and verotoxigenic *E. coli* (VTEC) in raw milk and traditional dairy cheeses marketed in Romania. total 200 samples, 95 isolates of *E. coli* strains from raw milk and 50 *E. coli* strains of telemy cheese were analyzed for virulence gene and resistance gene detection through PCR.

Badri *et al.* (2017) reported that 22 (38%) isolates had positive results in raw milk sample obtained from sudan.

Chaleshtori *et al.* (2017) conducted a study to determine *E. coli* isolated from traditional milk products from local markets in Kashan, Iran. A total of 116 samples were collected and the incidences of *E. coli* were 8.33%, 10% and 11.54%, respectively, in 60 ice cream, 30 yogurt and 26 cheese samples.

Disassa *et al.* (2017) analyzed 380 raw milk samples collected from a Town, in Western Ethiopia. Out of the 380 raw milk samples, *E. coli* was isolated from 129 (33.9%) samples based on morphological and cultural characteristics and biochemical tests.

Ombarak *et al.* (2018) analyzed 222 *E. coli* isolates from 187 dairy products, including 72 raw milk samples, 55 Karish cheese and 60 Ras cheese were collected from Nile delta region, Egypt. Of these, 55 raw milk samples (76.4%), 41 Karish cheese (74.5%) and 13 Ras cheese (21.7%), respectively, show the highest percentages of *E. coli* isolates (111, 89%) and the lowest percentages (111, 22%).

Ranjbar *et al*. (2018) investigated the prevalence of *E. coli* strains in different types of milk samples and dairy products obtained from Isfahan province, Iran. Out of 600 samples, 181 (or 30.16%) tested positive for *E. coli*. The percentage of *E. coli* found in cheese (80%) and raw buffalo milk (50%) samples was the greatest, while that of raw camel milk (6.66%) was the lowest.

Jhandai *et al.* (2019) investigated the occurrence of *E. coli* in various foods of animal origin from the Hisar district in Haryana, India. According to the findings, 74/130 (56.92%) of the samples tested positive for *E. coli*. *E. coli* was recovered from 14/30 (46.67%) samples of raw milk, 13/20 (65%) samples of pasteurized milk and 6/30 (20%) samples of ice cream.

Mahanti *et al.* (2020) detected the prevalence of *E. coli* in 45.6% of raw milk samples from West Bengal, India. Out of 450 samples, 205 tested positive for *E. coli*.

Ansharieta *et al.* (2021) assessed microbiological quality of 200 samples of raw cow milk in East Java, Indonesia. Based on the morphological growth of the colonies on the EMB Agar and the biochemical IMViC tests, the study's findings revealed that 70.5% of the isolates were identified as *E. coli*.

Jindal *et al.* (2021) examined 153 of raw pooled milk samples collected from small, medium and large farms located in all seven tehsils of Ludhiana district of Punjab. Study revealed that the prevalence of *E. coli* was determined to 60%, respectively, out of 153 samples of raw pooled milk.

Liu *et al.* (2021) studied 195 raw milk samples of the milk of dairy cattle in Northern China and 67 (34.4%) of the samples tested positive for *E. coli*.

Sultana *et al.* (2021) investigated 150 milk and milk products (yogurt) samples collected from the Bangladeshi Rajshahi Metropolitan region. *E. coli* was present in raw milk at a prevalence of 26.0% and in milk products (yogurt) at a prevalence of 34.0%, respectively.

Eldesoukey *et al.* (2022) examined 150 milk samples for the prevalence of EPEC in three dairy farms in Egypt. In the study, all isolates were assessed using repetitive extragenic palindromic sequence-based PCR (REP-PCR). EPEC isolates were detected in 5.3% (8/150) of milk samples.

Ibrahim *et al.* (2022) examined 200 samples of raw milk, white soft cheese, yoghurt and laban rayeb in different localities in Mansoura city of Egypt. The prevalence of *E. coli* was found to be 28% (56/200) from collected samples. The highest prevalence was detected in raw milk (52%) from market, followed by Kareish cheese (48%) while, the lowest prevalence was observed in yogurt and laban rayeb samples (8%).

Madani *et al.* (2022) investigated the prevalence of *E. coli* in milk and dairy products in Isfahan, Iran. A total of 200 samples were analyzed and 54 *E. coli* positive samples were detected, including (48/110) and (6/90) traditional and pasteurized dairy product samples.

Tyasningsih *et al.* (2022) collected 250 raw milk samples from five dairy farms in East Java, Indonesia. Study revealed the prevalence of *E. coli* was 176 (70.4%).

2.5. Antibiogram of *E. coli*

E. coli antimicrobial resistance has been detected by (Sabate *et al*., 2018). Their finding revealed due to the development of resistance to the majority of first-line antimicrobial drugs, the management of *E. coli* infections were more difficult to treat.

Farzana *et al.* (2005) detected the antibiotic sensitivity of *E. coli* isolates from raw milk in Multan, Pakistan. They found *E. coli* isolate was highly sensitive to Ceftriaxone, Tetracycline, Nalidixic acid and Chloramphenicol.

Thaker *et al.* (2012) examined 100 samples of raw milk from Anand, Gujrat, 38 of which had *E. coli*. Their antibiogram pattern showed great resistance to ampicillin (100%) but only moderate resistance to streptomycin (57.89%), oxytetracycline (47.37%) and amoxy-clav (47.37%). Resistance to co-trimoxazole (13.16%) and chloramphenicol (5.26%) was also lower.

Gundogan and Avci (2013) estimated the antibiogram of *E. coli* isolates isolated from foods of animal origin in Turkey. Isolates were 100% susceptible to

imipenem and cefepime but 100% resistant to ampicillin, tetracycline (77.8%), cefotaxime (33.3%), ciprofloxacin (31.1%), aztreonam (28.9%), ceftazidime (8.9%) and gentamicin (6.7%).

Bhoomika *et al.* (2015) conducted the study and performed the antibiotic sensitivity test for *E. coli* isolates, which were found resistant to cefotaxime (41.36%), followed by oxytetracycline (34.03%), ampicillin (29.31%), cephalexin (24.60%), cefixime (16.75%) and ceftazidime (13.08%). These strains were isolated from raw milk samples obtained from Chhattisgarh.

Disassa *et al.* (2017) screened antibiogram patterns for *E. coli* isolates from raw cow milk of Western Ethiopia. In study isolates were found resistant to tetracycline (81.8%), streptomycin (81.8%), kanamycin (63.6%), cefoxitin (54.5%) and norfloxacin (54.5%).

Batabyal *et al.* (2018) conducted an AST of the ESBL-positive *E. coli* isolates from milk samples West Bengal, India revealed that all the isolates were susceptible to antibiotic drugs such as colistin (100%), levofloxacin (83.33%) and imipenem (66.67%) but drugs like tetracycline (75.00%), ceftacycline (100%), ceftazidime (91.67%), amoxicillin/clavulanic acid (83.33%) and gentamicin (58.33%) all are highly resistant.

Effendi *et al.* (2018) studied the antibiogram pattern of *E. coli* isolates from raw milk samples from East Java Province, Indonesia. A total of 150 milk samples were collected and assessed, revealing a higher resistance against erythromycin (100%) and gentamicin (26%), followed by oxytetracycline (17.78%) and chloramphenicol (0%).

Kumar *et al.* (2018) screened for antimicrobial susceptibility patterns of *E. coli* isolated from milk and dairy products collected from Chennai. A total of 567 samples, 34 *E. coli* isolates were positive and studied revealing resistance to penicillin-G and cephalothin (94.11%) followed by ampicillin (88.23%), amoxicillin (82.35%) and clindamycin (76.47%). Among these 34 *E. coli* isolates, 28 (82.35%) were multiple drug resistant (MDR).

Ombarak *et al.* (2018) examine antimicrobial resistance in 222 isolates of *Escherichia coli* from 187 samples of raw milk and raw milk cheeses in Egypt. In a study revealed the 222 *E. coli* isolates, 66 (29.7%) were resistant to one or more antimicrobials and half of these resistant isolates showed a multidrug resistance. The resistance were observed to tetracycline (27.5%), ampicillin (18.9%), streptomycin (18.5%), sulfamethoxazole-trimethoprim (11.3%), cefotaxime (4.5%), kanamycin (4.1%), ceftazidime (3.6%), chloramphenicol (2.3%), nalidixic acid (1.8%) and ciprofloxacin (1.4%).

Elbehiry *et al.* (2021) studied 33 *E. coli* that were tested against various antibiotics in Al-Qassim region, Saudi Arabia. According to its findings, *E. coli* isolates were found sensitive to cefazolin, ceftazidime, cefotaxime, ceftriaxone and cefepime, in that order: 78.79% (26/33), 66.67% (22/33), 60.61% (20/33), 54.55% (18/33) and 39.40% (13/33), respectively.

Sultana *et al.* (2021) investigated antibiotic sensitivity patterns for isolated *E. coli* obtained from Bangladesh. An investigation demonstrated 100%, 60%, 40%, 40%, 33.3%, 20.0% and 10.0% resistance to penicillin, gentamycin, ampicillin, streptomycin, Amoxycillin, sulfamethoxazole-trimethoprim, nalidixic

acid and ciprofloxacin, respectively. Additionally, the isolates were sensitive to ciprofloxacin, nalidixic acid, sulfamethoxazole-trimethoprim, streptomycin, Amoxycillin, ampicillin and gentamycin in proportions of 73.3%, 60.0%, 53.3%, 53.3%, 30.0%, 23.3% and 20%, respectively.

Hassani *et al.* (2022) conducted a study of antibiotic susceptibility tests performed on *E. coli* isolates in the northwest of Iran. This investigation showed by a phenotypic method that all *E. coli* strains were highly resistant to penicillin (86.46%), cefalexin (82.05%) and amoxicillin (70.51%).

Imre *et al.* (2022) conducted a study in Romania, *E. coli* isolates showed resistance to enrofloxacin (100%), ampicillin (17/43), norfloxacin (28.6%), fosfomycin (25%), amoxicillin/clavulanic acid (23.3%), cefalexin (20%), cefalotin (13.3%), tetracycline (13.3%), trimethoprim-sulfamethoxazole (9.3%), piperacillin-tazobactam (7.1%), cefotaxime (7.1%), cefepime (7.1%), ceftazidime (3.6%), ertapenem (3.6%), ticarcillin/clavulanic acid (100%) and florfenicol (6.7%). Ten (23.3%) strains were resistant to multiple drugs.

Mahdavi *et al.* (2022) estimated the antibiotic sensitivity pattern of *E. coli* isolated from raw milk in Khorasan Razavi province, Iran. According to the susceptibility test results, less than 20% of *E. coli* isolates displayed intermediate resistance to any antibiotic. 3.33% had intermediate resistance to ciprofloxacin and levofloxacin, 15% to enrofloxacin and norfloxacin and 16.7% to ofloxacin.

Tyasningsih *et al.* (2022) screened *E. coli* isolates that were subjected to antibiotic susceptibility tests in raw milk samples obtained from raw milk in East

Java, Indonesia. *E. coli* isolates were found to exhibit resistance to antibiotics such as tetracycline (17.05%), streptomycin (14.2%), trimethoprim (9.7%), chloramphenicol (7.9%) and aztreonam (1.7%) isolates.

Widodo *et al.* (2022) studied Antibiotic sensitivity profile of multidrug-resistant (MDR) *E. coli* isolated from dairy cow's milk in Probolinggo, Indonesia. A total of 150 milk samples were obtained, the results reported that 124/150 (82.67%) *E. coli* bacteria exhibited highest percentage of antibiotic resistance to tetracycline (13.71%), followed by streptomycin (9.68%), trimethoprim (8.87%), chloramphenicol (0.87%) and aztreonam (1.61%). A total of 9/124 (7.26%) *E. coli* isolates were detected as multidrug-resistant (MDR)

2.6. Phenotypic characterization of *E. coli* isolates for ESBL

Bhoomika *et al.* (2015) found in Chhattisgarh, out of 73 *E. coli* isolates obtained from milk, 9 (12.32) isolates were phenotypically identified as presumptive ESBL producers.

Batabyal *et al.* (2018) reported in West bengal, India, a total of 12 (54.54%) *E. coli* isolates were found to be phenotypically positive for ESBL producers by the double disc method.

2.7. Molecular characterization of of *E. coli* isolates for ESBL (*bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$) genes

Skockova *et al.* (2014) examined the prevalence of ESBL-producing *E. coli* isolates obtained from raw milk samples in Brno, Czech Republic. In

investigation, 20 (7.4%) isolates were resistant to beta-lactam antibiotics, with *bla*$_{TEM}$ (6.6%) being the most prevalent, 2(0.7%) isolates of the *bla*$_{CTX-M}$ gene and none of the isolates identified for *bla*$_{SHV}$ gene.

Su *et al.* (2014) detected extended-spectrum β-lactamase-producing *E. coli* isolated from milk in Southern Taiwan. The *bla*$_{TEM}$ gene was detected (47%), *bla*$_{CTX-M}$ [*bla*$_{CTX-M3}$-like (9%) and *bla*$_{CTX-M14}$-like (4%)] and *bla*$_{SHV}$ (9%).

Badri *et al.* (2017) discovered the prevalence of the ESBL-producing *E. coli* isolated from raw milk in Sudan. The ESBL-encoded CTX-M gene was reported 61%, followed by the SHV gene 23% and the TEM gene 16%.

Tabaran *et al.* (2017) examined *E. coli* isolates for the beta-lactamase resistance gene obtained from Romania. A total of 9 (33.3%) isolates showed positive results for the *bla*$_{TEM}$ gene and none of the samples tested positive for *bla*$_{SHV}$ genes.

Batabyal *et al.* (2018) conducted in West Bengal to determine that all phenotypically ESBL-positive *E. coli* isolates were detected to have the *bla*$_{CTX-M}$ gene by PCR.

Yang *et al.* (2018) discovered the prevalence of the ESBL gene *E. coli* isolated from bovine mastitis cases in China. Detected Extended-spectrum β-lactamase producers were 55 (73.3%) isolates of the quinolone-resistance gene. While *bla*$_{CTX-M}$ and *bla*$_{SHV}$ were found in 35 (46.7%) and 2 (2.7%) isolates, respectively, the dominant β-lactamase gene, *bla*$_{TEM}$, was recognized in 44 (58.7%) isolates.

Kamaruzzaman *et al.* (2020) discovered in Malaysia, 18 ESBL-producing *E. coli* isolates. 8 out of 18 (44.4%) isolates had a predominant ESBL genotype that was a mix of TEM and CTX-M, according to 2 (11.1%) isolates produced the TEM gene, 4 (22.2%) isolates produced ESBL genes other than TEM, SHV, CTX-M and four (22.2%) isolates produced the CTX-M gene.

Ahmed *et al*. (2021) determined the prevalence of ESBL-producing *E. coli* isolated from raw milk in Iraq. In this study, 28.75% (23/80) isolates of raw milk samples were positive for ESBL resistance gene.

Ansharieta *et al.* (2021) reported that 2.12% (3/141) of the *E. coli* isolates produced ESBLs from raw cow's milk in East Java, Indonesia. The PCR results showed that the double $bla_{\text{CTX-M}}$ and bla_{TEM} gene harbored by 2 ESBL isolates and one bla_{TEM} gene as many as 1 ESBL isolate.

Liu *et al.* (2021) examined that 40 *E. coli* isolates with beta-lactam resistance had a prevalence of 45% in China. In total, 1.5, 20.9 and 1.5% of having the bla_{SHV}, bla_{TEM} and $bla_{\text{CTX-M}}$ genes.

Younis *et al.* (2021) detected ESBL producing *E. coli* isolates from raw milk samples collected from Egypt. In an investigation, 10 *E. coli* isolates were positive for the bla_{TEM} gene.

Eldesoukey *et al.* (2022) conducted a study in ESBL isolates isolated from milk samples in Iran. This study showed 37.5% of isolates for bla_{SHV} and bla_{TEM} genes, followed by 12.5% of isolates that showed the $bla_{\text{CTX-M 1}}$ gene.

Hassani *et al.* (2022) collected 100 raw bovine milk samples from different retail sellers in northwest Iran. A total of 39 (50%) *E. coli* isolates tested positive for *bla*$_{TEM}$, while 5 (6.41%) tested positive for *bla*$_{SHV}$. The most prevalent resistance gene in the *E. coli* isolates identified in this study was *bla*$_{TEM}$.

Saei *et al*. (2022) detected an ESBL-producing *E. coli* isolated from raw milk sample in Iran. The study revealed the highest prevalence of *bla*$_{TEM}$ (46.87%) and *bla*$_{CTX-M}$ -14 (43.75%) genes by the Multiplex PCR method.

Tyasningsih *et al.* (2022) analyzed 176 *E. coli* isolates collected from five dairy farms in East Java, Indonesia. A study identified 3 (1.7%) ESBL producers and all 3 (100%) ESBL-producing *E. coli* isolates harbored *bla*$_{TEM}$ genes.

2.8. Prevalence of antibiotic resistance genes in *E. coli* isolates

Skockova *et al.* (2014) detected the prevalence of tetracycline resistance genes prevalence in *E. coli* isolates obtained from raw milk in Brno, Czech Republic. In the investigation, the *tet*A gene was more frequently identified (7.0%) than the *tet*B gene (5.5%).

Tabaran *et al.* (2016) determined the prevalence of the tetracycline resistance gene in isolates obtained from raw milk and unpasteurized traditional cheeses in Romania. A total of 13 (48.1%) isolates showed positive results for the tetracycline resistance genes, with 8 (61.5%) isolates of *tet*A having the highest prevalence.

Pyatov *et al.* (2018) investigated resistance genes in *E. coli* isolated in milk samples from dairy farms in the Vysocina Region, Brno, Czechia. A total of 72 samples were obtained and this study revealed A tetracycline resistance was *tet*A 14 (19.4%) and *tet*B 21 (28.2%) of the isolates identified.

Yang *et al.* (2018) studied the quinolone-resistant *E. coli* isolated from bovine mastitis cases in China. Out of 328 *E. coli* isolates from 2,954 mastitic milk samples, 75 (22.9%) isolates harbored ciprofloxacin-resistant gene.

Ranjbar *et al.* (2018) examined antibiotic-resistance genes of *E. coli* isolates from raw milk and traditional dairy product samples in Isfahan province, Iran. The study revealed a prevalence of 76.56% of *tet*A and 20.31% of *tet*B resistance genes.

Younis *et al.* (2021) examined a total of 100 milk samples collected from different sources (dairy farms, retail markets and farmers' houses) in Qena, Egypt. In this study, they found that all 10 isolates of *E. coli* were positive for the *tet*A and 4 isolates of *E. coli* isolates were positive for the *tet*B gene, according to this study the tetracycline resistance genes *tet*A and *tet*B were the most prevalent.

CHAPTER III
MATERIALS AND METHODS

The present study was conducted in the Department of Veterinary Public Health and Epidemiology, College of Veterinary Science and Animal Husbandry, Anjora, Durg, Chhattisgarh. The study was deals on isolation, identification, biochemical screening, and antibiotic sensitivity test and molecular characterization of *E. coli* from milk and milk products. Samples were collected from Durg, Raipur, Bilaspur and Rajnandgaon districts of Chhattisgarh.

3.1 Materials

3.1.1 Glass wares and plastic wares

The glass wares used in the present study were procured from Borosil Glass wares Ltd. India, whereas, plastic wares and other disposables were procured from Tarson Product Pvt. Ltd. India. The glasswares were washed and sterilized following the standard procedures and used during the study.

3.1.2 Media, chemicals and reagents

All the bacteriological media, chemicals, and reagents used in the present study were obtained from Hi-Media, India, Thermo Scientific, USA, and Bangalore Genei, India and prepared according to the instructions provided by the manufacturing firms and were checked for sterility before use. The details of use of culture media, reagents, stains, etc. throughout the study given in appendix.

3.1.3 Equipments and instruments

Autoclave (Obromax), Deep freezer (Remi), Electronic balance (Sartorius), PCR (Thermocycler Applied Biosystems^{TM} Proflex^{TM}, USA), Nanodrop (Thermo fisher, USA), Gel documentation system (Biorad, USA),

Horizontal gel electrophoresis unit (Biometra, Germany), Hot air oven (Unitech), Incubator (Weiber), Refrigerated Centrifuge (Thermo Fisher),UV transilluminator (Biometra TI3), Laminar flow (Klenz flow), Micropipette (Thermo Scientific), Refrigerator (LG), Vortex mixer (Mac) and Water bath (Rivotek) were used during the present study.

3.1.4 Primers

The sequence and length of the primers targeting the 16S rRNA and resistance genes are given in table 1 and 2. All the primer are procured from Eurofins Scientific, Bengaluru, India.

Table 1: Details of the primer used for amplification of 16S rRNA gene.

Target gene	**Sequence of primer (5'-3')**	**Amplicon size (bp)**	**References**
16S rRNA	**F:** ATCAACCGAGATTCCCCCAGT	231	Sundong-bo *et al*.,2011
	R: TCACTATCGGTCAGTCAGGAG		

Table 2: Details of the primers used for amplification of resistance genes

Antimicrobial family	Target gene	Sequence of primer (5'-3')	Amplicon size (bp)	References
Beta-lactams	*bla*$_{SHV}$	**F :** ATGCGTTATATTCGCCTGTG	747	Paterson *et al.,* 2003
		R: TGCTTTGTTAT CGGGCCAA		
	bla$_{TEM}$	**F:** TCGCCGCATACACTATTCTCAGAATGA	445	Apaka *et al.,* 2010
		R: ACGCTCACCGGCTCCAGATTTAT		
	bla$_{CTX-M}$	**F:** ATGTGCAGYACCAGTAARGTK ATG GC	593	Boyd *et al*., 2004
		R: TGGGTRAARTARGTSACCAGAAYCAGC GG		
Tetracycline	*tet*A	**F:** GCTACATCCTGCTTGCCTTC	210	Titilawo *et al.,* 2015
		R: CATAGATCGCCGTGAAGAGG		
	*tet*B	**F:** TTGGTTAGGGGCAAGTTTTG	359	
		R: GTAATGGGCCAATAACACCG		
Fluroquinolone	*gyr*A	**F:** CGTCGCGTACTTTACGCCATGAACG	586	Dasgupta *et al.,* 2018
		R: ATACCTTGCCGCGACCGGTACGG		
	*par*C	**F:** TGTATGCGATGTCTGAACTG	265	
		R: CTCAATAGCAGCTCGGAATA		

3.1.5 Antibiotic discs:

Antibiotic discs from HiMedia Laboratory Pvt Limited, Mumbai were used in antibiotic sensitivity test.

3.2 Methods

3.2.1 Sample collection

A total of 200 samples comprising of raw milk (n=120), and milk products (n=80) were collected for isolation of *E. coli* (Table 3) from Durg, Raipur, Bilaspur, and Rajnandgaon districts of Chhattisgarh during the present study (Table 4). The samples were collected following the protocol recommended by International Commission on Microbiological Specification for Food (ICMSF, 1978). All the samples were collected immediately after milking in sterile bottles and transported to the laboratory under chilled conditions for analysis within 4-6 hrs.

Table: 3 Details of milk and milk products samples collected during the study

S.No.	Source of samples	No. of samples
1	Raw milk	120
2	Milk products	80
Total		**200**

Table: 4 District wise details of milk and milk products samples collected from Chhattisgarh

S.No.	Districts	No. of Raw milk sample	No. of milk products
1.	Durg	30	20
2.	Raipur	30	20
3.	Bilaspur	30	20
4.	Rajnandgaon	30	20
Total		**120**	**80**

3.2.2 Isolation of *E. coli* from raw milk and milk products samples

For isolation of *E. coli* from raw milk and milk products samples, standard ISO 16654:2001 protocols was followed with slight modifications. The following steps were performed. Raw milk and milk products samples were collected from dairies, hotels, restaurants, vendors and households. Each sample was collected aseptically into a sterile screw-capped bottle and kept in an ice box containing ice packs, and brought immediately to the laboratory for bacteriological analysis.

Enrichment

Each milk sample and milk products (dahi, lassi, buttermilk and paneer) sample was enriched in MacConkey's broth in a 1:10 ratio and incubated at 37°C for 18-24 hrs.

Selective plating

Loopful of broth culture from enrichment media was streaked on MacConky's agar (MLA) and incubated at 37ºC for 20- 24 hrs. The pink to red colored bacterial colonies on MLA were picked up and streaked onto the Eosine Methylene blue (EMB) agar plate. *E. coli* showed a metallic sheen around the colonies on EMB. *E. coli* colonies were further used to for identification.

3.2.3. Gram's staining

The representative *E. coli* colonies were characterized microscopically using Gram's stain according to the method described by Agrawal *et al*. (2003). Briefly, a small colony was picked up with a bacteriological loop, smeared on a glass slide and fixed by gentle heating. Crystal violet solution was added on the smear for one minute and then washed with running water. Lugol's iodine was added for one minute and then washed with running water. Alcohol was added for few seconds. After washing with water, safranine was added for one minutes. The slide was then washed with water, blotted and dried in air and then examined under microscope.

3.2.4. Biochemical characterization of *E. coli* isolates

The bacterial isolates were subjected to various biochemical tests. Isolated colonies from the selective agar plates were streaked onto nutrient agar slant and

incubated to various biochemical test *viz.* Indole test, Methyl red, Voges-proskauer and Citrate utilization test following the procedure described by Agrawal *et al.* (2003). For further identification, *E. coli* isolates were subjected to various biochemical tests as per the protocols described by Ewing *et al.* (1986). The Himedia biochemical kit KB002- HiAssorted^{TM} Biochemical Test Kit was also used for biochemical tests.

3.2.4.1 Indole test

In this test, the microorganism is incubated in tryptone broth (Himedia) at 37°C for 24 h followed by the addition of Kovac's reagent (Hi-media, Appendix). The appearance of the red colored ring was taken as positive.

3.2.4.2. Methyl-Red (MR) test

Presumptive isolates were inoculated in tubes containing 5ml of glucose phosphate broth (Hi-media). After 48 h incubation at 37°C, 5 drops of MR solution were added. The appearance of red color immediately was taken as positive for the MR test. In case of a negative result (yellowish-orange color), incubation of the broth was continued for an additional three days, and retesting of the broth culture was done.

3.2.4.3. Voges-Proskauer (VP) test

In this test also glucose phosphate broth (Hi-media) was used to test presumptive isolates. After 72 h incubation at 37°C, Barrit's reagent solution A and B (Hi-media) was added. The appearance of pink burgundy color was taken as positive for the VP test, and in case of negative reaction copper/yellow color was considered as negative.

3.2.4.4. Citrate utilization test

For citrate utilization, commercially available Simmon's citrate medium (Hi-media) was used. Slants were prepared and cultures to be tested were inoculated and incubated at 37°C for 24 hrs. Change in color from green to blue was taken as positive while no color change was taken as negative for citrate utilization.

3.2.5. Molecular confirmation for *E. coli*

3.2.5.1 Extraction of bacterial genomic DNA

Template DNA incorporated in the PCR reaction was prepared by boiling and snap chill methods (Nagappa *et al.,* 2007). Briefly, culture from nutrient agar was inoculated into 5 ml of Luria-Bertani broth (LB) and incubated for 12-16 hrs at 37ºC. Cells from 1.5 ml of the culture were harvested by centrifugation at 10000 rpm in a microcentrifuge for 2 min at room temperature. The supernatant was decanted and the pellet in a microcentrifuge tube was added with 1.5 ml phosphate buffer saline and mixed by vortexing, the suspension was then centrifuged for 2 min and the supernatant was discarded. The pellet was mixed with 300µl nuclease-free water and then put into a water bath for 15 min at 100°C followed by immediate chilling on crushed ice for at least 20 min. Finally, tubes were centrifuged at 12000 rpm for 2 min and the clear supernatant was collected and stored at -20°C until further use.

3.2.5.2.Measurement of DNA concentration and purity

The concentrations of DNA were measured with Nanodrop 2000C (Thermo Scientific, USA), and adjusted to 100 ng/µl for further PCR studies. Pure

DNA samples (with an optical density ratio of 1.8 to 2 at 260/280 nm) were stored at – 20°C, until further use (Desjardins and Conklin, 2010).

3.2.5.3. PCR conditions for detection of *E. coli* (16S rRNA)

PCR reactions were carried out in a Thermocycler (Applied Biosystems™ Proflex™, USA). The amplification conditions were 3 min of denaturation at 94 °C, followed by 35 cycles of 94°C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 30 seconds, and a final extension step of 72 °C for 10 min (Sundong-bo *et al.,* 2011). The PCR reaction mixture (10µl) contains primers (forward 0. 5 µl and reverse 0. 5 µl), master mix (red taq ready mix) 5 µl, molecular grade water 2 µl and genomic DNA 2 µl.

3.2.6. Antibiotic sensitivity test (AST)

An antibiotic sensitivity test was performed as per the method of Bauer and Kirby (1966). Commercially available antibiotic discs (HiMedia laboratories Limited, Mumbai) were used to test the susceptibility of the isolated *E. coli* against different antibiotics listed in table 5.

For determining the susceptibilities of bacterial isolates to different antibiotics, disc diffusion method was performed on Muller- Hinton agar (MHA) plates and results were interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI,2020). Pure culture from the nutrient agar slant was transferred into a tube containing 5 ml LB broth. The broth culture was incubated at 37 °C for 24hr. After incubation, a sterile cotton swab was dipped into the suspension. The swab was swirled several times and pressed firmly on the clear wall of the tube to remove excess inoculum. The inoculum in swab was then

inoculated on the dried surface of a Mueller -Hinton agar plates by rubbing the swab over the entire sterile agar surface. This procedure was repeated two more times, rotating the plates approximately 60° each time to ensure an even distribution of inoculum. The plate was left for 3 to 5 min to allow any excess surface moisture to be absorbed before applying the antibiotic impregnated discs plates.

Table: 5 Antibiotic discs used in the present study

S.N.	Antibiotic discs	Symbol	Concentration (mcg)	Interpretative criteria diameter of zone of inhibition (mm)		
				R	I	S
01.	Cephalexin	CN	30	<14	-	>14
02.	Oxytetracycline	O	30	<11	12-14	>15
03.	Amoxyclav	AMC	30	<13	14-17	>18
04.	Gentamicin	GEN	10	<12	13-14	>15
05.	Ciprofloxacin	CIP	5	≤15	16- 20	≥21
06.	Cefotaxime	CTX	30	<22	23-25	>26
07.	Amoxycillin	AMX	30	<13	14- 17	>18

R= Resistant, I =intermediate, S= sensitive

3.2.6.1 Application of discs to inoculated agar plates

The predetermined set of antimicrobial discs *viz.* Cephalexin (30 mcg), Oxytetracycline (30 mcg), Amoxyclav (30 mcg), Gentamicin (10 mcg), Ciprofloxacin (5 mcg), Cefotaxime (30 mcg), Amoxycillin (30 mcg) were placed onto the surface of the inoculated agar plates. The discs were distributed evenly with a minimum gap of 24 mm from centre to centre. The plates were placed in an inverted position in an incubator at 37°C within 15 min of the placement of the discs. After 16 to 18 hrs of incubation, each plate was examined for the zones of inhibition. The diameter of the zones of complete inhibition was measured and compared with the zone size interpretation chart provided by manufacturers and were graded as sensitive, intermediate, and resistant.

3.2.7. Multiple antibiotic resistance (MAR) index

The MAR index for each *E. coli* isolate was calculated by applying a/b where "a" is the number of antibiotics to which an isolate was resistant and "b" is the number of antibiotics to which the isolates were exposed (Krumperman, 1985).

3.2.8. Phenotypic test for identification of presumptive ESBL producers

3.2.8.1 Double Disc Diffusion Test (DDDT)

The phenotypic test for presumptive ESBL producers was performed as per CLSI guidelines (CLSI, 2020). For this purpose, Cefotaxime (30µg), and Cefotaxime-Clavulanic acid (30/10µg), discs were used. Discs were placed 25 mm apart on a MHA plate inoculated with 0.5 McFarland suspension of the test isolate. Plates were incubated at 37°C for 18 hrs. After incubation, the zone diameter around each disc was measured. A difference of ≥ 5mm between the

zone diameters of either of the cefotaxime discs and their respective cefotaxime / clavulanic acid discs was considered a positive phenotypic test for ESBL producers (Rawat and Nair, 2010; Kazemian *et al*.,2019).

3.2.8.2 Hichrome ESBL Agar test

Hicrome ESBL agar (Himedia, Mumbai, India) is based on an antibiotic-mixture agar that is specifically designed to support the selective growth of *Enterobacteriaceae* that produce ESBL. On this medium, the bacterial colony were inoculated, and they were immediately incubated for 18 to 24 hours at 37°C. Blue or Pink colonies were recorded as presumptive positive ESBL-producing *E. coli* (Devi *et al.*,2021).

3.2.9. Amplification of 16S rRNA and resistance genes by PCR

The *E. coli* isolates which were identified as presumptive ESBL producers and those showed resistance against were screened for ESBL (bla_{TEM}, bla_{CTX-M} and bla_{SHV}) and *tet*A, *tet*B, *gyr*A, *par*C genes respectively. For amplification of the various gene 10 µl volume reaction mixtures consisting of the following components was used.

Table 6: Details of the primers used for amplification of genes

Content	Molecular grade water (µl)	Master mix2X (Red Taq Ready mix) (µl)	Primers (Forward and Reverse) (µl)	Genomic DNA (µl)	Total Reaction volume (µl)
***bla*SHV**	1	5	0.5/0.5	1	10
***bla*TEM**			0.5/0.5		
***bla*CTX-M**			0.5/0.5		
***tet*A**	1.5	5	0.5/0.5	1.5	10
***tet*B**			0.5/0.5		
***gyr*A**	1.5	5	0.5/0.5	1.5	10
***par*C**			0.5/0.5		

3.2.9.1 Amplification of *bla*SHV, *bla*TEM and *bla*CTX-M gene

PCR targeting *bla*$_{SHV}$, *bla*$_{TEM,}$ and *bla*$_{CTX-M}$ genes was performed as per the method described by Apaka *et al.* (2010). The cycling conditions were optimized for the PCR reaction mixture which consisted of an initial denaturation (95°C for 10 min) followed by 30 cycles of denaturation (94°C for 30s), primer annealing (60°C for 30s), and extension (72°C for 2 min). A final extension at 72°C was given for 10 min. The PCR products thus obtained were kept at 4°C and used in electrophoresis.

3.2.9.2 Amplification of *tet*A and *tet*B gene

PCR targeting *tet*A and *tet*B genes was performed as per the method described by Titilawo *et al.* (2015). The cycling conditions were optimized for the

PCR reaction mixture which consisted of an initial denaturation (95°C for 5 min) followed by 35 cycles of denaturation (95°C for 30s), primer annealing (59°C for 30s), and extension (72°C for 1 min). A final extension at 72°C was given for 5 min. The PCR products thus obtained were kept at 4°C and used in electrophoresis.

3.2.9.3 Amplification of *gyr*A and *par*C gene

PCR targeting *gyr*A and *par*C gene was performed as per the method described by Dasgupta *et al.* (2018). The cycling conditions were optimised for PCR reaction mixture which consisted of an initial denaturation (95°C for 2 min) followed by 32 cycles of denaturation (95°C for 25s), primer annealing (52°C for 1m) and extension (72°C for 1.2 min). A final extension at 72°C was given for 7 min. The PCR products thus obtained were kept at 4°C and used in electrophoresis.

3.2.10 Agarose gel electrophoresis

To analyse the amplified products for 16s rRNA and resistance genes in *E. coli* isolates the submarine agarose gel electrophoresis was performed as described by Russell and Sambrook (2001). Agarose gel (1.6%) was prepared by putting 0.8 gm agarose in 50 ml 1XTris Borate EDTA (TBE) buffer and subjected to heat until the agarose was completely dissolved and appeared as a clear transparent solution. The agarose solution was allowed to cool to 60°C and then ethidium bromide (0.5µg/ml) dye was added to it. Thereafter the gel was poured into the gel casting tray held within the gel holding tray and the comb was placed into the slots on the tray in such a manner that a gap of 0.5 mm was left between the tips of comb teeth and the floor of casting tray so that the wells were

completely sealed by the agarose. It was allowed to solidify for 20-30 minutes and then the comb was gently removed. The casting gel along with the running tray was submerged into electrophoresis tank containing 1X TBE buffer. A total volume of 8µl PCR products were taken on a clean parafilm and mixed evenly with 2µl of 6X gel loading dye (Thermo scientific Ltd.) and loaded carefully into the wells of agarose gel. To determine the size of the amplified PCR product 100 bp DNA ladder was loaded in one well.

Electrophoresis was performed at 70 V for 45 min and the mobility was monitored by the migration of the dye in the gel. After appropriate migration, the gel was visualised under a UV transilluminator.

3.2.11 Visualization of the gel in Gel Doc^{tm} XR

The amplified PCR products were visualized under a transilluminator and the gel were documented by Gel Doc^{TM} XR Bio rad (USA).

CHAPTER IV

RESULTS AND DISCUSSION

In the present study, attempts were made to isolate and characterize *E. coli* from milk and milk Products. All *E. coli* isolates were confirmed by detecting16S rRNA gene and further tested against different antibiotics to obtain their multidrug resistance pattern. The prevalence of ESBL genes (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$) and resistance genes (*tet*A, *tet*B, *gyr*A and *par*C) among *E. coli* isolates was also determined.

4.1. Isolation and Identification of *E. coli*

In the present study, milk and milk product samples were processed to isolate and identify *E. coli*. A total of 200 milk samples comprising of raw pooled milk (n=120) and milk products (n=80) were collected randomly from retail dairies, hotels, restaurants, vendors, and households located in different parts of Durg, Raipur, Bilaspur, and Rajnandgaon districts of Chhattisgarh.

Samples were inoculated in MacConkey Lactose broth medium for enrichment process, and thereafter a loopful of culture from enrichment broth was streaked onto selective media MLA and EMB. Isolate showing pink, moist colony on MLA and metallic sheen around the colonies on EMB were identified as *E. coli* and subjected to further identification (Fig. 1 and 2).

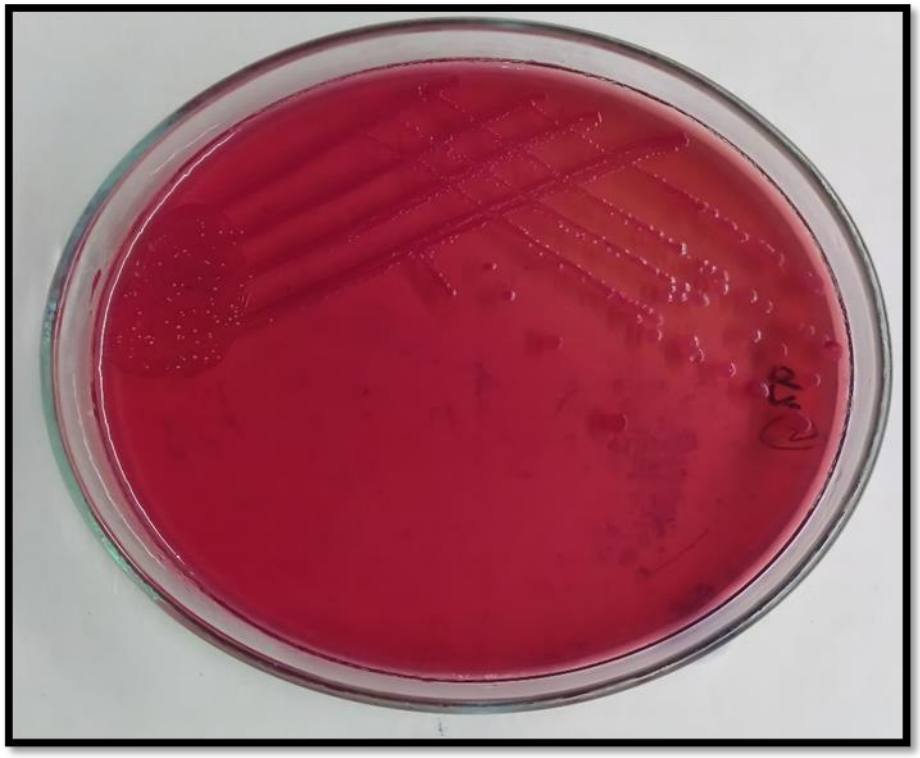

Fig: 1 *E. coli* isolates showing pink, moist colony on MacConkey's Lactose Agar (MLA) plate

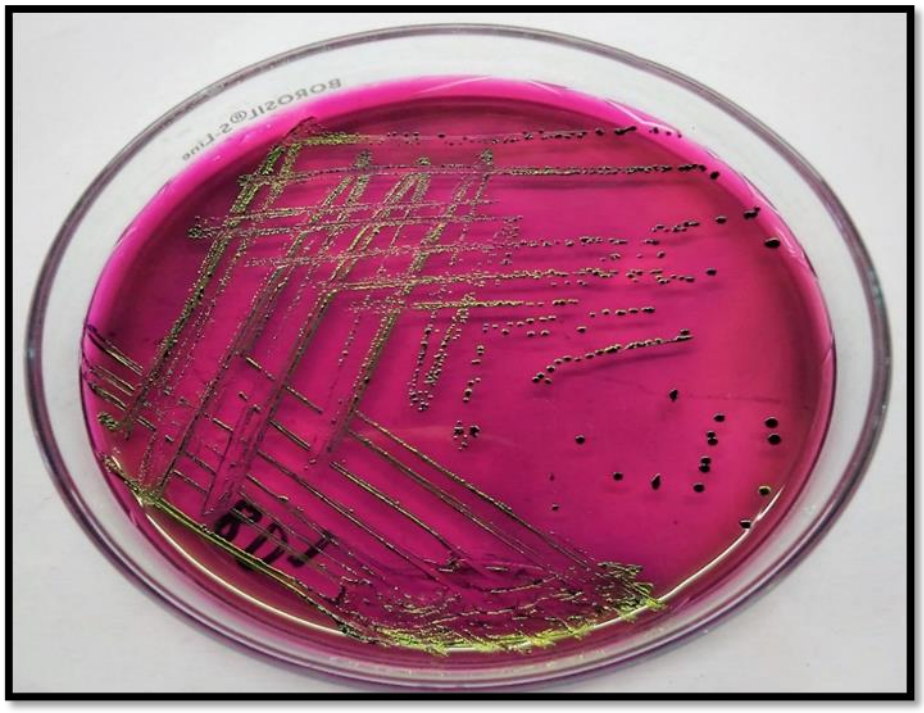

Fig: 2 *E. coli* isolates showing metallic sheen around the colonies on Eosin Methylene Blue (EMB) agar

4.2. Gram's staining

In gram's staining under microscope, the organism's revealed gram negative, pink colored, small rod shaped appearance, arranged in single, paired or chain form (Fig. 3).

4.3. Biochemical characterization of *E. coli* isolates

All the *E. coli* isolates were further subjected to biochemical tests as Indole, Methyl red (MR), Voges Proskauer (VP), Citrate utilization. *E. coli* isolates showed (+ + - -) IMViC pattern (Fig. 4 and 5), which is in accordance to the observations made by Sultana *et al.* (2021) in Bangladesh.

The biochemical tests were also performed by using KBM002 HiMedia Biochemical kit for *E. coli*. All the isolates of *E. coli* gave positive reaction for Lysin utilization, Ornithine utilization, Urease, Nitrate reduction, Glucose, Lactose, Arbinose and Sorbitol and negative reaction for Urease, Phenylalanine Deamination, H_2S production, Adonitol and Citrate utilization test.

4.4. Molecular confirmation of *E. coli* isolates

PCR results confirmed the presence of *E. coli* DNA in 63 isolates using housekeeping gene primers (16S rRNA) (Fig. 6). Isolation of *E. coli* from milk samples was also reported in Egypt reported (Younis *et al*., 2021) and Romania (Imre *et al*., 2022).

4.5. Prevalence of *E. coli*

A total of 63 *E. coli* isolates were recovered from 200 raw milk and milk products samples with the overall prevalence of 31.5%. The higher prevalence of 38.33% (46/120) was recorded in raw milk samples as compared to 21.25%

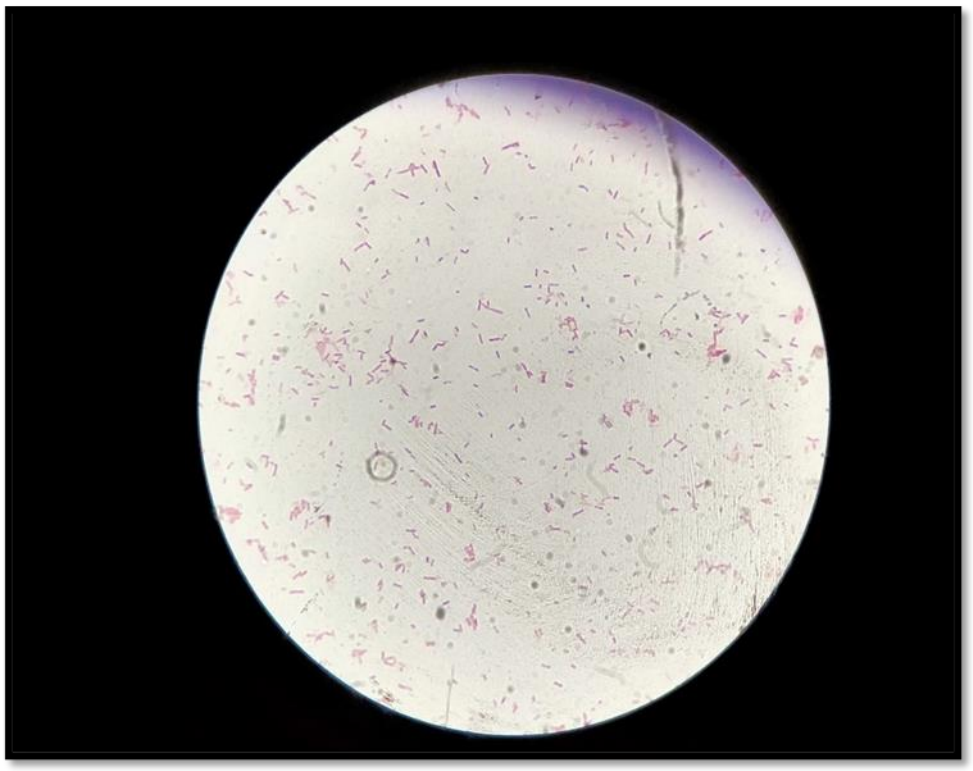

Fig: 3 *E. coli* showing Gram-negative, small rod-shaped appearance, arranged in single or paired.

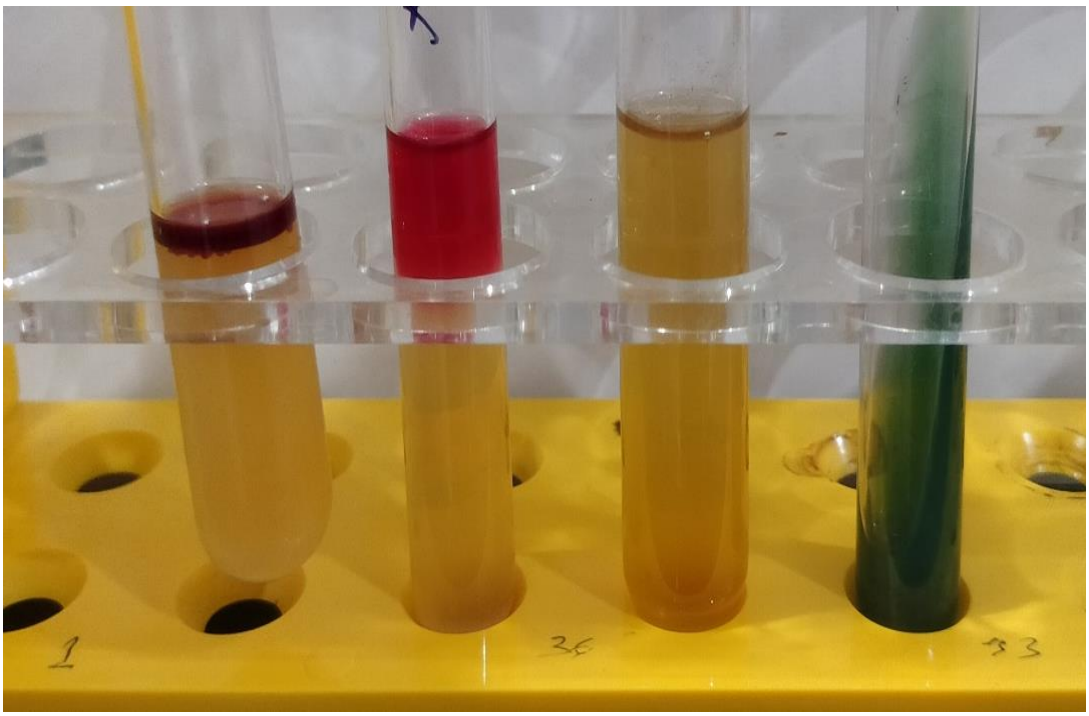

Fig. 4: *E. coli* showing biochemical IMViC test, (Indole - Positive, Methyl Red -Positive, Voges-Proskauer - Negative, Citrate test -Negative)

Fig. 5: Biochemical test kit for *E. coli* isolates

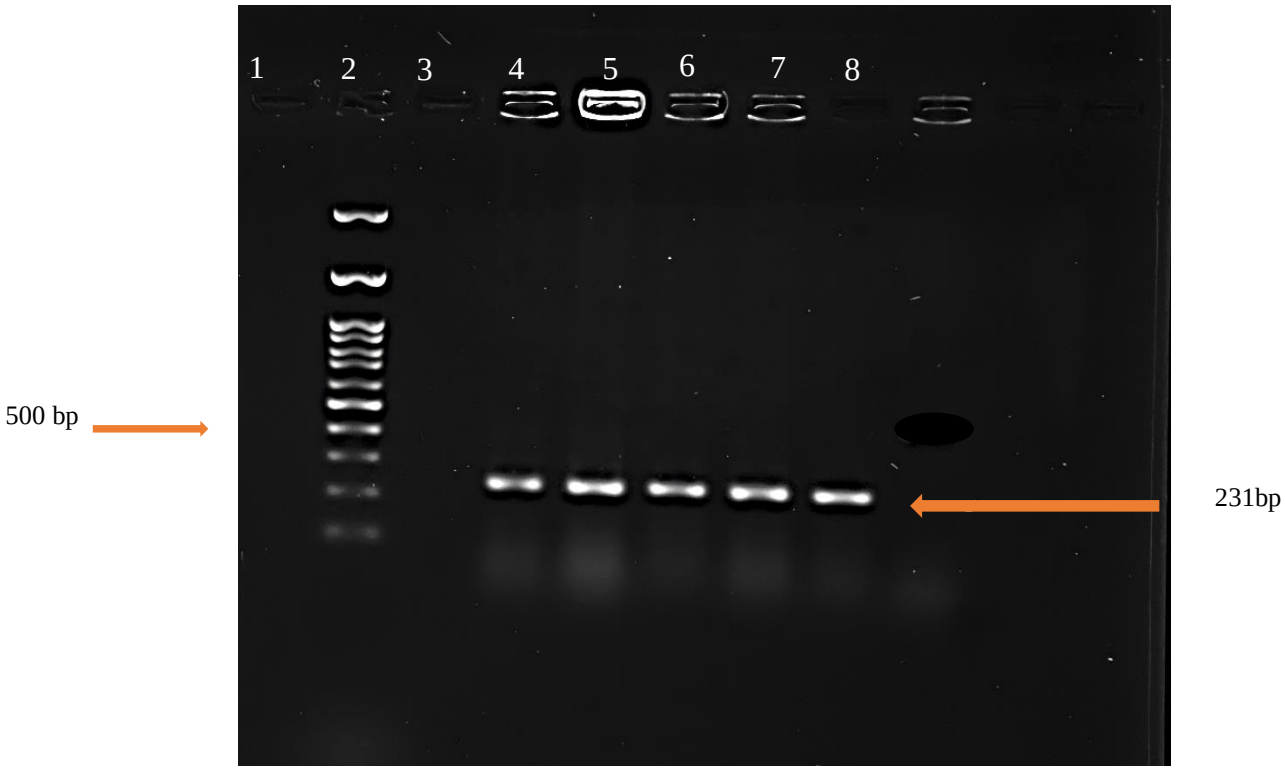

Fig. 6: Agarose gel electrophoresis showing amplified PCR product of 16S rRNA (231bp) for *E. coli.* Lane 1- 100bp ladder, Lane 3,4,5,6 Positive sample, Lane- 2,7 Negative sample

(17/80) in milk products samples (Table 7 and Fig 7). The details of prevalence recorded in milk products *viz*. dahi (8/25), lassi (7/20), buttermilk (1/20) and paneer (1/15) are given in Table 7.

The district wise prevalence of *E. coli* in raw milk and milk products samples is shown in Table 8 and Fig. 8. Among districts, Durg showed highest prevalence rate of *E. coli* in raw milk and milk products (40%) followed by Rajnandgaon (34 %) and least from Raipur and Bilaspur (24 %). The highest prevalence rate in raw milk was observed in Durg (46.66%) followed by Rajnandgaon (43.33%), Raipur (33.33%) and least from Bilaspur (30%). In milk products highest prevalence rate observed in Durg (30%) followed by Rajnandgaon (20%), and (20%) for Raipur and Bilaspur districts in Chhattisgarh.

In the present study prevalence of *E. coli* in raw milk was observed 38.33% which is in agreement with the findings of Thaker *et al.* (2012) who reported prevalence rate of 38.00% in raw milk in Gujrat, India. Badri *et al.* (2017) also reported 38% prevalence in raw milk obtained from Aljazira state, Sudan. Higher prevalence rate of 81.1% from raw milk in Chhattisgarh was also reported (Bhoomika *et al.,* 2016). Similarly Ombarak *et al.* (2016) reported 76.4% prevalence of *E. coli* in raw milk from Egypt and 60% was also reported by Jindal *et al.* (2021). In India 46.67% prevalence of *E. coli* in raw milk reported by Jhandai *et al.* (2019). Mahanti *et al.* (2020), reported prevalence of *E. coli* 45.6% in milk. Joseph and Kalyanikutty (2022) was also reported prevalence of *E. coli* 47.16% in raw milk and Ibrahim *et al.* (2022) respectively, from Egypt. However lower prevalence rate was reported by the Batabyal *et al.* (2018*)*, who reported

12.08% prevalence of *E. coli* in samples. 26.0% in raw milk from Rajshahi metropolitan area of Bangladesh observed by Sultana *et al.* (2021). The 34.4% prevalence of *E. coli* in raw milk in China was reported by Liu *et al.* (2021). The 24.8% prevalence of *E. coli* was recorded in raw milk in Punjab, India (Jindal *et al.,* 2021), Younis *et al*. (2021), reported a prevalence of 10% *E. coli* in the milk samples in Egypt. In contrast, a much lower incidence (5.3%) of *E. coli* was discovered in milk samples in Egypt (Eldesoukey *et al.,* 2022)

Results revealed the 21.25% prevalence of *E. coli* in milk products. However, lower prevalence rate of 34.0% in milk products was reported in Rajshahi metropolitan area of Bangladesh (Sultana *et al.,* 2021). Higher prevalence rate of 81 % of *E. coli* in milk products from Romania was reported by Imre *et al.* (2022) and 34% was reported by Bajrami and Sulaj (2017) and Ombarak *et al.* (2016) respectively, from Egypt and Soomro *et al.* (2002) reported 51.66% highest rate of prevalence in milk products samples from Pakistan.

Table 7: Prevalence of *E. coli* in raw milk and milk products

S. No	Source	No. of samples analyzed	No. of samples tested positive	
1.	Raw milk	120	46 (38.33%)	
2	Milk products	80		17 (21.25%)
	1. Dahi (n= 25)		8	
	2. Lassi (n= 20)		7	
	3. Buttermilk (n=20)		1	
	4. Paneer (n=15)		1	
Total		200	63 (31.5%)	

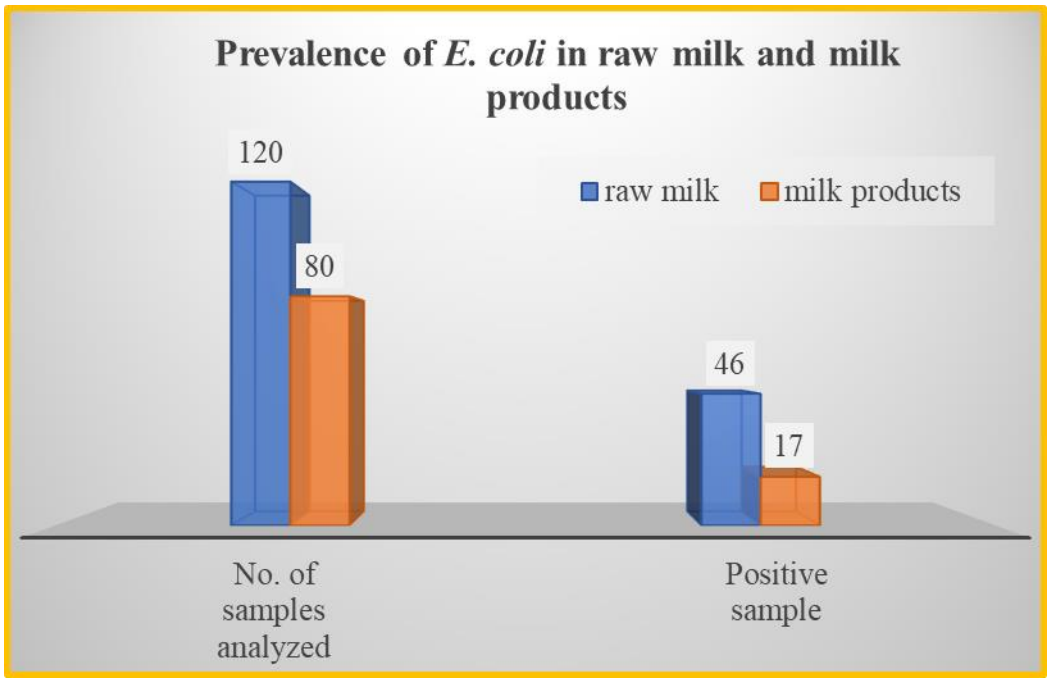

Fig. 7: Prevalence of *E. coli* in Raw milk and milk products samples

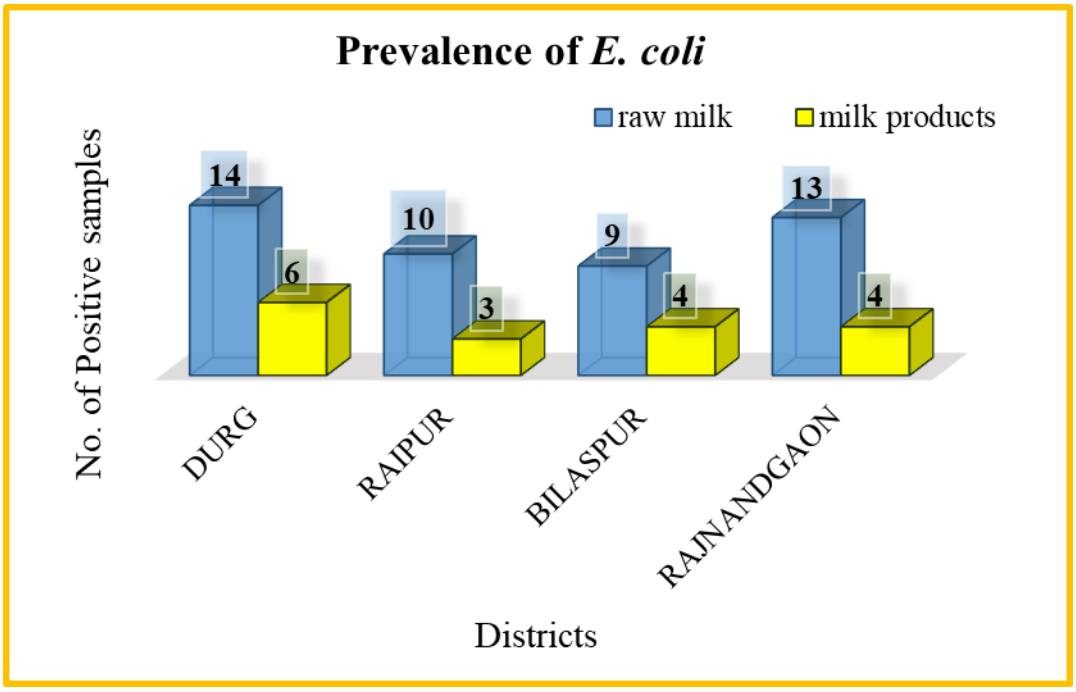

Fig. 8: Prevalence of *E. coli* in raw milk and milk products in different districts of Chhattisgarh

Table 8: Prevalence of *E. coli* in raw milk and milk products in different districts of Chhattisgarh.

S.No.	Districts	Raw milk		Milk products		Total
		No.of samples analysed	No. of samples positive (%)	No.of samples analysed	No. of samples positive (%)	
1	Durg	30	14 (46.66%)	20	6 (30%)	20 (40%)
2	Raipur	30	10 (33.33%)	20	3 (15%)	13 (24%)
3	Bilaspur	30	9 (30%)	20	4 (20%)	13 (24%)
4	Rajnandgaon	30	13 (43.33%)	20	4 (20%)	17 (34%)
Grand Total		**120**	**46 (38.33%)**	**80**	**17 (21.25%)**	**63 (31.5%)**

4.6. Antimicrobial susceptibility testing

The Antimicrobial susceptibility testing of all identified *E. coli* isolates were done according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2020) (Fig. 9 and 10).

The 63 *E. coli* isolates from raw milk and milk products were exanimated by the disk diffusion method for their susceptibility against 7 antibiotics. Antibiotic resistance in *E. coli* isolates was observed to Amoxycillin (88.88%), Amoxyclav (87.30%), Cephalexin (74.60%), Cefotaxime (58.73%), Gentamicin (22.22%), Oxytetracycline (20.63%), and Ciprofloxacin (19.04%) (Table 9). Results revealed that out of 63 *E. coli* isolates, 49 (77.77%) were found to be

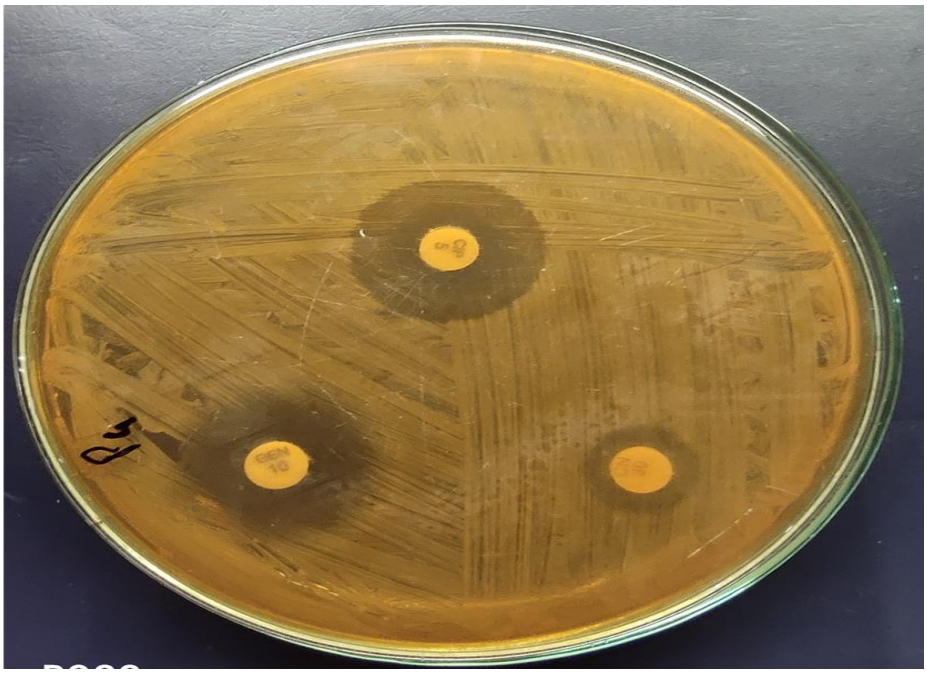

Fig. 9: Antibiotic Sensitivity Test showing the Zone of inhibition against different antibiotics.

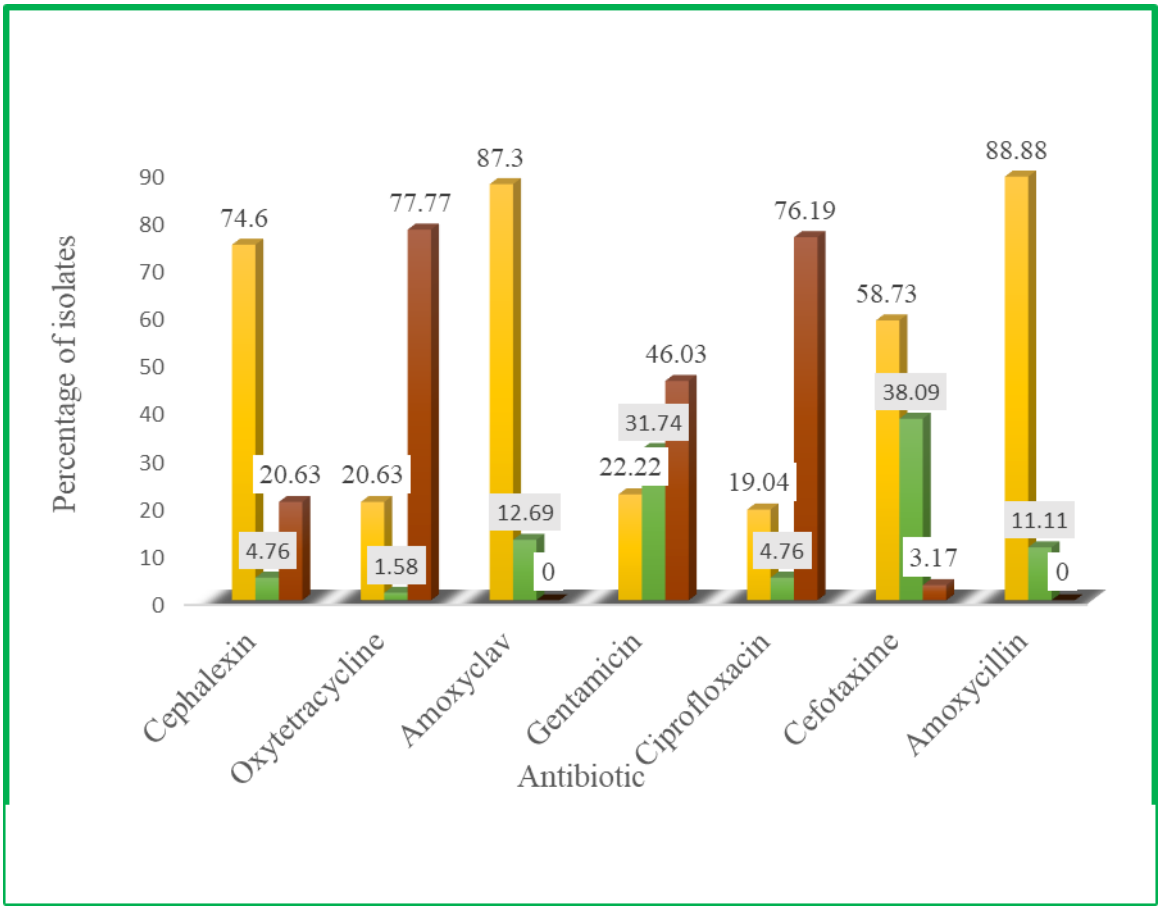

Fig. 10: Antibiogram pattern shown by *E. coli* isolates

sensitive for Oxytetracycline followed by 48 (76.19%) for Ciprofloxacin, 29 (46.03%) for Gentamicin, 13(20.63%) for Cephalexin and 2 (6.34%) for Cefotaxime. Whereas, none of the isolates showed sensitivity to these against Amoxyclav and Amoxycillin. Results further revealed that Out of 46 *E. coli* isolates from raw milk, 42 (91.30%) isolates showed the highest resistance to Amoxyclav, followed by 39 (84.78%) for Amoxycillin , 32 (69.56%) for Cephalexin , 30 (65.21%) for Cefotaxime, 11 (23.91%) for Gentamicin, 10 (21.73%) for Ciprofloxacin, and 8 (17.39%) for Oxytetracycline. For milk products isolates, 17 (100%) isolates showed highest resistance to Amoxycillin followed by 15 (88.23%) isolates for Cefalexin, 13 (76.47%) for Amoxyclav, 7 (41.17%) for Cefotaxime, 5 (29.41%) for Oxytetracycline, 3 (17.64%) for Gentamicin and 2 (11.76%) for Ciprofloxacin (Table 9 and 10).

Similar finding were reported by Hassani *et. al.* (2022) who reported that *E. coli* isolates were highly resistant to Cefalexin and Amoxycillin and Thaker *et. al.* (2012) reported that *E. coli* isolates were moderately resistance to Oxytetracycline and Amoxyclav. Kumar *et. al.* (2018) reported that *E. coli* isolates were resistance to amoxycillin in India. Imre *et al.* (2022), recorded resistance to cefalexin 20% and cefotaxime 7.1% in Romania.

Table 9: Antibiogram assay of *E. coli* isolates

S.No.	Antibiotic disc	Antibiotic sensitivity pattern (n=63)		
		Resistant	**Intermediate**	**Sensitive**
1.	Cephalexin	47 (74.60%)	3 (4.76%)	13(20.63%)
2.	Oxytetracycline	13 (20.63%)	1 (1.58%)	49 (77.77%)
3.	Amoxyclav	55 (87.30%)	8 (12.69%)	0 (0.0%)
4.	Gentamicin	14 (22.22%)	20 (31.74%)	29 (46.03%)
5.	Ciprofloxacin	12 (19.04%)	3 (4.76%)	48 (76.19%)
6.	Cefotaxime	37 (58.73%)	24 (38.09%)	2 (6.34%)
7.	Amoxycillin	56 (88.88%)	7 (11.11%)	0 (0.0%)

n= total number of isolates tested

Table 10: Percentage of antimicrobial resistance among the *E. coli* isolates from raw milk and milk products

S.No.	Antimicrobials disc	Raw milk (n=46)	Milk products (n=17)
1	Cephalexin	32 (69.56%)	15 (88.23%)
2	Oxytetracycline	8 (17.39%)	5 (29.41%)
3	Amoxyclav	42 (91.30%)	13 (76.47%)
4	Gentamicin	11 (23.91%)	3 (17.64%)
5	Ciprofloxacin	10 (21.73%)	2 (11.76%)
6	Cefotaxime	30 (65.21%)	7 (41.17%)
7	Amoxycillin	39 (84.78%)	17 (100%)

n= total number of isolates tested

4.7. Multiple antibiotic resistance (MAR) index

In the present study among 63 *E. coli* isolates, highest MAR index of I was observed for (2 isolates) followed by 0.85 (3 isolates), 0.71 (10 isolates), 0.56 (20 isolates), 0.42 (15 isolate), 0.28 (11 isolates) and 0.14 (2 isolates) (Table 11).

District wise in Durg district, out of 20 isolates, 1 isolate showed 0.71 MAR index, 3 isolates showed 0.56, 6 isolate showed 0.42, 8 isolates showed 0.28, 2 isolates showed 0.14 and 0.85 and1 isolate showed zero MAR index. In Raipur district, out of 13 isolates, 2 isolates were showed MAR index 0.71, 4 isolates showed 0.56, 5 isolates showed 0.42, 2 isolates showed 0.28 and zero MAR index was recorded for 0.14, 0.85, and 1. From Bilaspur district, out of 13 isolates, 3 isolates showed 0.71 MAR index, 7 isolates showed 0.56 MAR index, 3 isolates showed 0.42 MAR index and 0.14, 0.28, 0.85 and1 showed zero MAR index. In Rajnandgaon district, out of 17 isolates, 2 isolates showed MAR index 1,

3 isolates showed 0.85, 4 isolates showed 0.71, 6 isolates showed 0.56, 1 isolate showed 0.42, 1 isolate showed 0.28 and zero MAR index was recorded for 0.14 (Table 12). Previously work has been done on milk samples by Bhoomika *et al.* (2016) who reported MAR index ranging between 0 to 0.90.

Table 11: Pattern of antibiogram and MAR index for *E. coli* isolates

S.N.	Isolates	Antibiotic discs							MAR index
		CN	O	AMC	GEN	CIP	CTX	AMX	
1	DM1	I	S	R	S	R	S	R	0.42
2	DM3	R	S	R	R	I	R	I	0.57
3	D3M3	R	S	I	R	R	S	R	0.57
4	D1M4	S	S	R	S	S	R	I	0.28
5	DM8	S	S	R	I	S	I	I	0.14
6	DM10	S	S	R	S	S	I	I	0.14
7	DM11	S	S	R	I	S	R	R	0.42
8	DM12	R	R	R	S	S	R	R	0.71
9	DM14	R	S	R	S	S	I	R	0.42
10	DM20	R	S	I	S	S	I	R	0.28
11	DM23	S	S	R	I	S	I	R	0.28
12	DM24	R	S	R	I	S	I	R	0.42
13	DL2	R	S	I	S	S	I	R	0.28
14	DL4	S	S	R	I	S	I	R	0.28
15	DD4	R	S	R	I	S	I	R	0.42

16	DP5	R	S	I	S	S	I	R	0.28
17	DP8	S	S	R	I	S	I	R	0.28
18	DP9	R	S	R	I	S	I	R	0.42
19	DP12	R	S	I	S	S	I	R	0.28
20	DP13	R	S	R	I	S	R	R	0.57
21	KD1	R	S	R	S	S	I	R	0.42
22	RU1	S	S	R	S	S	I	R	0.28
23	RU2	S	S	R	S	S	R	R	0.42
24	RK1	R	S	R	I	S	R	I	0.42
25	RHC4	R	S	R	S	S	R	R	0.57
26	NYC1	R	S	I	S	S	R	R	0.42
27	NYB1	S	S	R	S	S	S	R	0.28
28	N1B2	R	S	R	I	S	R	R	0.57
29	RH2	R	S	R	S	S	I	R	0.42
30	PD5	R	R	R	S	S	R	R	0.71
31	RM1	R	S	R	S	R	I	R	0.57
32	RM4	R	R	R	S	S	R	R	0.71
33	RM8	R	S	R	S	R	R	R	0.57
34	BMT1	R	S	R	I	S	R	R	0.57
35	BD1	R	S	I	I	S	R	R	0.42
36	BL1	R	S	R	R	S	I	R	0.57
37	BT2	R	S	R	R	S	R	R	0.71
38	BL3	R	S	I	I	S	R	R	0.42

39	BE2	R	S	R	R	S	R	R	0.71
40	BL4	R	S	R	R	S	I	I	0.42
41	BJT3	R	S	R	R	S	I	R	0.57
42	BL6	R	S	R	R	S	I	R	0.57
43	TBC2	S	I	R	R	S	R	R	0.57
43	TBC2	S	I	R	R	S	R	R	0.57
44	BSP3	R	S	R	R	S	I	R	0.57
45	BSP6	R	S	R	R	S	R	R	0.71
46	B1D1	R	S	R	I	S	R	R	0.57
47	RJ1	I	S	R	S	S	R	I	0.28
48	RJ2	R	S	R	S	R	R	R	0.71
49	RJ3	R	S	R	I	S	R	R	0.57
50	RJ4	S	R	R	S	S	R	R	0.57
51	RJ8	R	R	R	S	S	R	R	0.71
52	RJ11	R	R	R	R	R	R	R	1
53	RJ19	I	S	R	I	R	I	R	0.42
54	RJ21	R	S	R	S	R	R	R	0.71
55	RP1	R	R	R	S	S	R	R	0.71
56	RP4	S	R	R	R	R	R	R	0.85
57	RP5	R	R	R	S	R	R	R	0.85
58	RP6	R	R	R	S	S	I	R	0.57
59	RP9	R	S	R	I	I	R	R	0.57
60	RP10	R	R	R	S	R	R	R	0.85

61	RP12	R	R	R	I	S	I	R	0.57
62	RP14	R	R	R	R	R	R	R	1
63	RP15	R	S	R	I	I	R	R	0.57

MAR=Multiple antibiotic resistance

R=Resistant, I=Intermediate, S=Sensitive

CN= Cefalexin, O=Oxytetracyclin, AMC=Amoxyclav, GEN=Gentamicin, CIP=Ciprofloxacin, CTX=Cefotaxime, AMX=Amoxyicillin

Table 12: MAR index for *E. coli* isolates from various districts of Chhattisgarh

S.N.	**Districts**	**MAR index for *E. coli* isolates**							**Total isolates**
		0.14	**0.28**	**0.42**	**0.56**	**0.71**	**0.85**	**1**	
1	Durg	2	8	6	3	1	0	0	20
2	Raipur	0	2	5	4	2	0	0	13
3	Bilaspur	0	0	3	7	3	0	0	13
4	Rajnandgaon	0	1	1	6	4	3	2	17
Total		2	11	15	20	10	3	2	63

4.8. Detection of ESBL producing *E. coli*

4.8.1. Phenotypic Detection of ESBL producers

A higher degree of multidrug resistance among *E. coli* isolates could be linked to their ability of ESBL production. CLSI (2020) Disc diffusion method was performed Mullar Hinton Agar (MHA) to detect presumptive ESBL producer among *E. coli* isolates using Cefotaxime (30 µg) and Cefotaxime with Clavulanate (30/10 µg). A difference of ≥ 5mm between the zone diameters of either of the cefotaxime discs and their respective cefotaxime / clavulanic acid discs were considered as positive phenotypic confirmatory test for ESBL producers. (Fig.11)

For selective screening of ESBL-producing bacteria, they were also inoculated onto hichrome ESBL agar base and its isolates showed dark pink to a purple colony were considered as presumptive ESBL producers (Fig. 12).

In the study, 25 (39.68%) isolates were phenotypically identified as presumptive ESBL producers. Among the *E. coli* isolates in raw milk, 45.65% (21/46) and milk products 23.52% (4/17) were phenotypically found positive for ESBL. Similarly, in research in Colombia, 6.6% of *E. coli* isolates were identified as ESBL from milk (Vasquez-Jaramillo *et al.,* 2016). In a study from India, it was revealed that 54.54% of *E. coli* isolates were phenotypically positive for ESBL producers (Batabyal *et al.,* 2018).

4.8.2. Molecular detection of ESBL

The genotypic techniques of PCR help in recognizing the genes $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} that encode for β-lactamases. The ESBL enzymes TEM, SHV, and CTX-M break down a wide range of cephalosporins, including ceftazidime

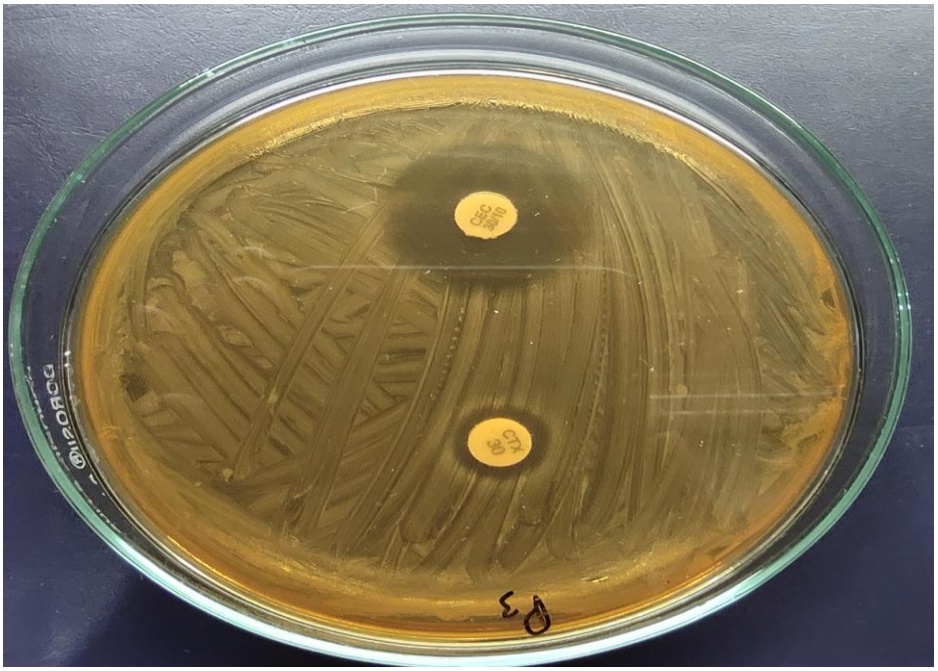

Fig. 11: Isolates of *E. coli* showing phenotypic confirmatory test for ESBL production

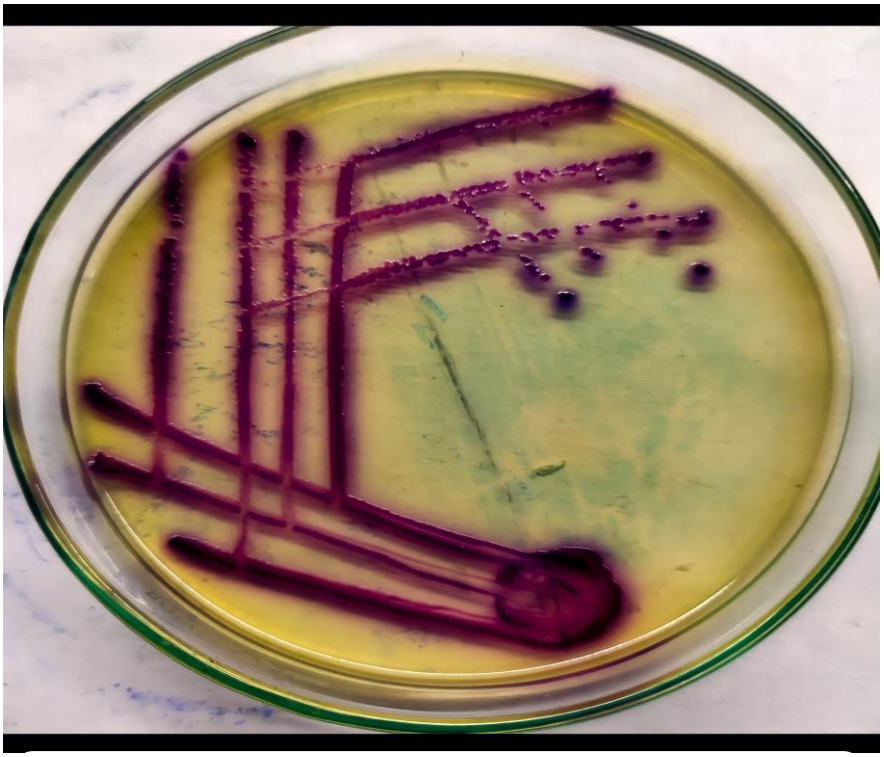

Fig. 12: Dark pink to purple colony on ESBL agar plate

and cefotaxime, as well as ampicillin, carbenicillin, and oxacillin. In this study multiplex PCR was performed to simultaneously identify two or more genes in a single isolate.

Out of 63 *E. coli* isolates 8 (12.69%) isolates were displayed expression of bla_{TEM} and 9 (14.28%) isolates displayed expression of $bla_{\text{CTX-M}}$. whereas, 8 (12.69%) isolates expressed the bla_{SHV} gene (Fig. 14 and 15). In *E. coli* Isolates obtained from raw milk 7 (15.21%) isolates expressed bla_{TEM} gene, 8 (17.39%) isolates expressed $bla_{\text{CTX-M}}$ gene and 6 (13.04%) isolates expressed bla_{SHV}. From milk products, only 1 (5.88%) isolate expressed bla_{TEM} gene and $bla_{\text{CTX-M}}$ and 2 (11.76%) isolates expressed bla_{SHV} genes (Fig. 13, 14 and Table 13).

In previous study, Hassani *et al.* (2022) reported that high prevalence bla_{TEM} (50%) and bla_{SHV} (6.41%) were detected in *E. coli* isolates obtained from Iran. Liu *et. al. (*2021) reported 1.5, 10.4, and 20.9% prevalence of $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} genes, respectively in China. Younis *et. al*. (2021) reported that all 10 isolates of *E. coli* were positive for the bla_{TEM} in Egypt. Ahmed, (2021) also reported 84.61% of CTX- M gene in *E. coli* isolated in Iraq. Badri *et al.* (2017) revealed 61%, 23%, and 16% prevalence of CTX- M, SHV, and TEM genes, respectively in Sudan. Batabyal *et.al.* (2018) reported a 54.54% ESBL -producers *E. coli* isolates in milk from India. Tekiner *et. al.* (2015) showed 75% and 100% ESBL prevalence of *E. coli* from raw milk and milk cheese in Turkey and A high prevalence of ESBL-producing *E. coli* in milk (66.7%) was reported by Kamaruzzaman *et al*. (2015). Bhoomika *et al.* (2016), reported the 3.66% of bla_{TEM} and 2.09% of $bla_{\text{CTX-M}}$ but none of the isolates found positive for bla_{SHV}, study also reports *bla*-encoded enzyme TEM, was more prevalent than SHV among *E. coli* isolates.

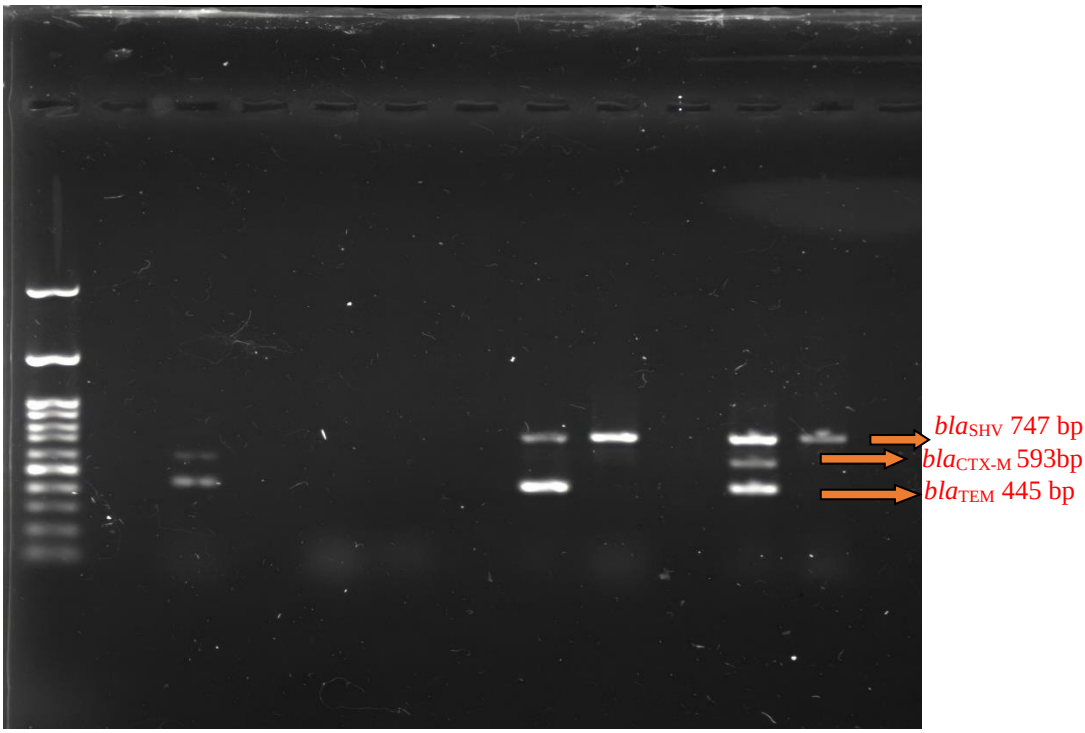

Fig. 13: Agarose gel showing *bla*SHV, *bla*CTX-M and *bla*TEM genes amplified from *E. coli* isolates. Lane 1: 100 bp ladder, Lane 2: Negative control, Lane 3: Positive control, Lane 4,5,6,7: negative

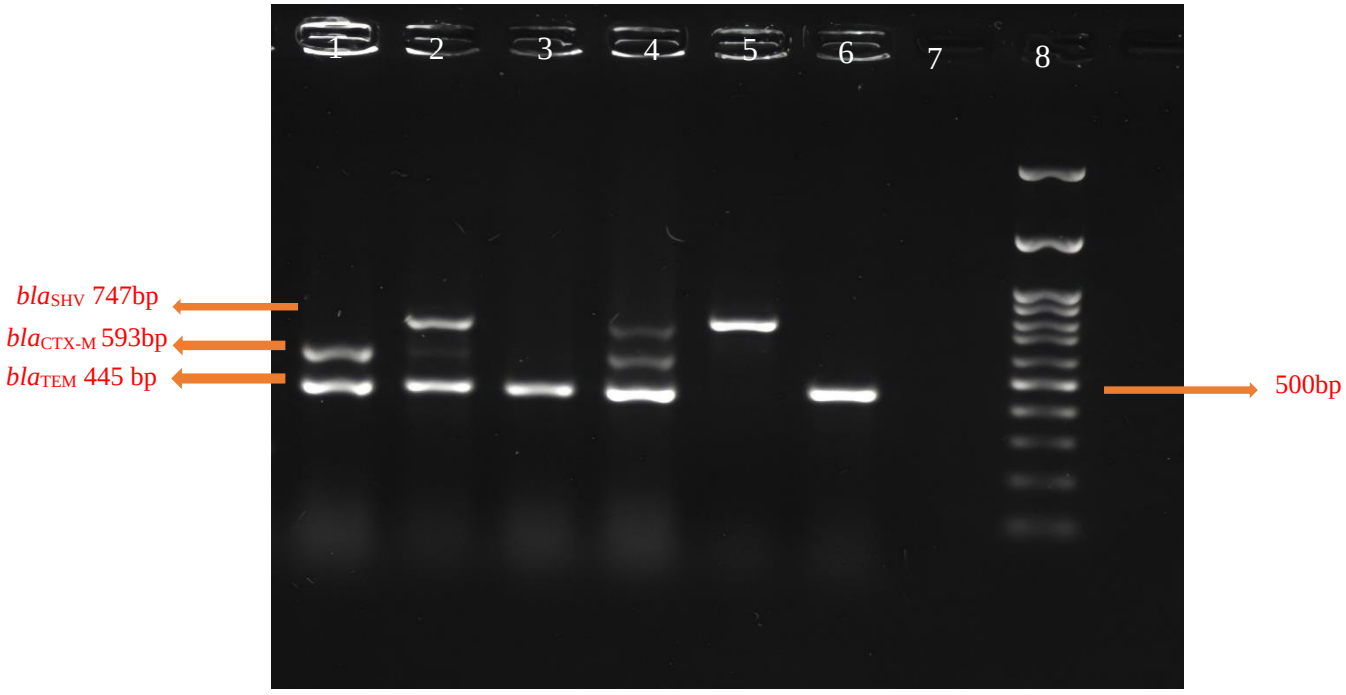

Fig. 14: Agarose gel electrophoresis shows *bla*SHV, *bla*CTX-M and *bla*TEM genes amplified from *E. coli* isolates. Lanes 1-6: Test samples, Lane 7: Negative sample, Lane 8: 100 bp ladder.

Table 13: Prevalence of ESBL genes in *E. coli* isolates

Type of samples	ESBL producer (%)			
	Phenotypic method	Molecular method		
		bla_{TEM}	bla_{CTX-M}	bla_{SHV}
Raw milk (n=46)	21 (45.65%)	7 (15.21%)	8 (17.39%)	6 (13.04%)
Milk products (n=17)	4 (23.52%)	1 (5.88%)	1 (5.88%)	2 (11.76%)
Total (n=63)	25 (39.68%)	8 (12.69%)	9 (14.28%)	8 (12.69%)

4.9. Molecular detection of resistance genes

In the present study, among 13 Tetracycline resistant *E. coli* isolates, 7 (53.84%) harbored *tet*A gene whereas only 1(7.69%) isolates harbored *tet*B gene (Fig. 15 and Table 14). However, Younis *et al.* (2021) reported that 10 isolates of *E. coli* were positive for the *tet*A gene and 4 isolates were positive for the *tet*B gene in Egypt. Gomi *et al*. (2017) reported the prevalence of *tet*A was more than *tet*B in *E. coli* isolates. Tabaran *et. al.* (2016) reported that 61.5% of *tet*A and 53.8% of *tet*B were prevalent in raw milk and unpasteurized traditional cheeses in

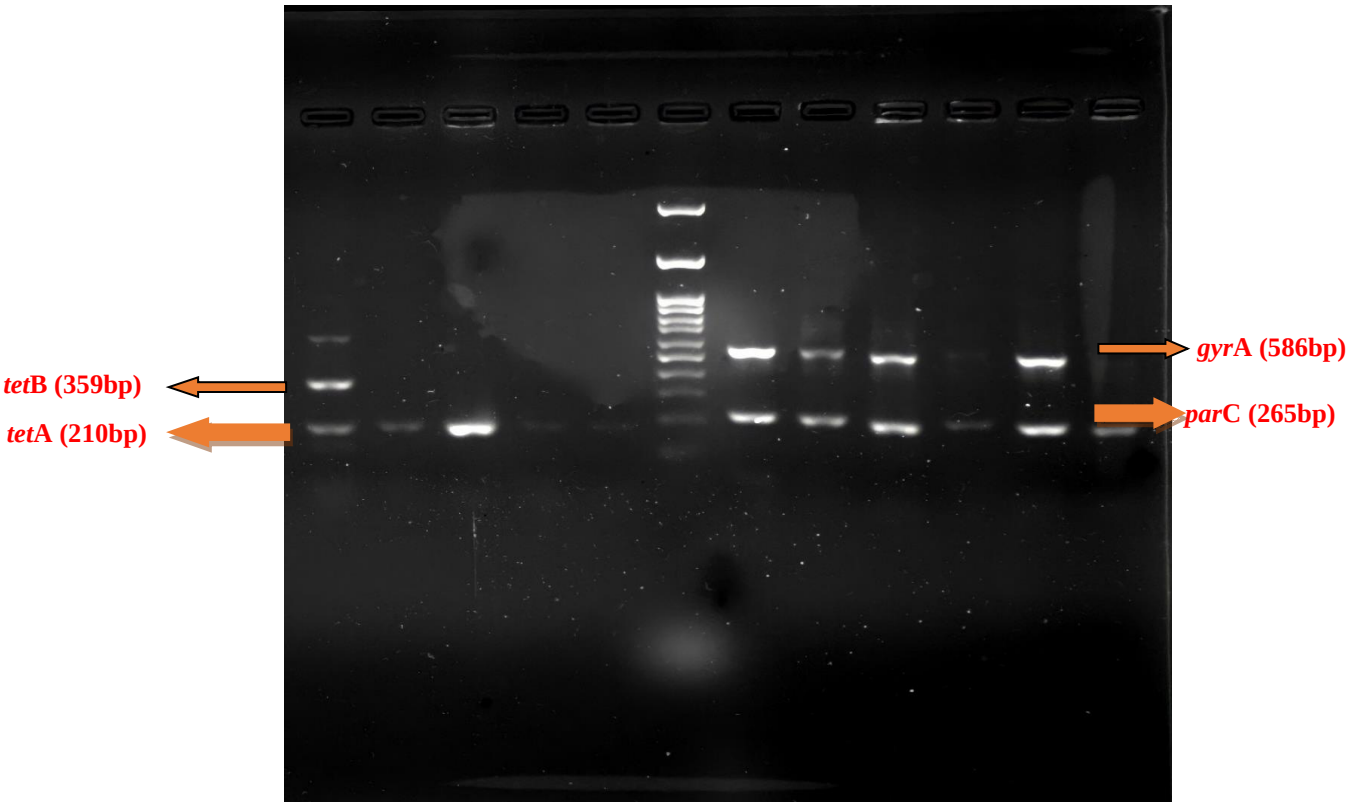

Fig. 15: Agarose gel electrophoresis showing PCR product of *tet*A gene and *tet*B gene left side and *par*C gene and *gyr*A gene right side for *E. coli* isolates

Romania. Pyatov *et. al.* (2015) *tet*A (19.4%) and *tetB* (28.2%) of the isolates was detected in milk in Brno Czech Republic.

Furthermore, among 12 Fluoroquinolones resistant *E. coli* isolates, 6 (50%) isolates expressed *par*C, Whereas, 4 (33.33%) isolates expressed the *gyr*A gene (Fig. 16 and Table 14). The studies in Thailand, its research detected genomic mutations in *gyr*A and *par*C Genes of *E. coli* by PCR (Onseedaeng and Ratthawongjirakul, 2016).

Table 14: Prevalence of resistance genes in *E. coli* isolates

Types of samples	**Resistant gene (%)**			
	Tetracycline (n=13)		**Fluoroquinolones (n=12)**	
	***tet*A**	***tet*B**	***gyr*A**	***par*C**
Raw milk	6 (46.15%)	1 (7.69%)	3 (25%)	5 (41.66%)
Milk products	1 (7.69%)	0	1 (8.33%)	1 (8.33%)
Total	**7 (53.84%)**	**1 (7.69%)**	**4 (33.33%)**	**6 (50%)**

n= number of *E. coli* resistant to respective antimicrobial groups

CHAPTER-V
SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FURTURE RESEARCH WORK

SUMMARY:

Food-borne diseases are significant causes of illness and death and continue to be a serious public health issue worldwide. Milk is a major part of human food and plays a prominent role in the human diet. Milk contains protein and calcium and is a good source of vitamin B_{12}, thiamine, and riboflavin. In India peoples traditionally consumes raw milk at small farms where it is produced or processed into a variety of foods. The hygienic measures are frequently not given enough concern. During these practices consumption of unpasteurized milk and milk products increases the risk of foodborne illness in consumers by 150 times. Antibiotics are regularly added to feed as feed additives in intensive animal production system at sub-therapeutic doses to stimulate growth, improve feed efficiency, and avoid infections. The extensive use of antibiotics in human treatment, animal therapy, and agricultural use as growth promoters are further causes of bacterial resistance to antibiotics. Due to their development of antibiotic resistance, gram-negative bacteria constitute a therapeutic challenge not only in medical settings but also in the general public. The high presence of bacteria that produce ESBLs and are multidrug-resistant in raw milk poses a possible health concern to anyone who consumes raw milk and milk products.

In present study, a total of 200 samples (120 raw milk and80 milk products samples) were collected from Durg, Raipur, Bilaspur and Rajnandgaon districts of

Chhattisgarh and analyzed for the isolation and identification of *E. coli* by conventional cultural technique and further confirmed by biochemical test. All isolates were confirmed by detecting 16S rRNA gene of *E. coli*. Then *E. coli* isolates were further tested against different antibiotics to obtain their multidrug resistance pattern. The prevalence of ESBL genes (*bla*CTX-M, *bla*SHV, *bla*TEM) and resistance genes (*tet*A, *tet*B, *gyr*A and *par*C) among *E. coli* isolates were confirmed by molecular techniques.

A total of 63 *E. coli* isolates were recovered from raw milk and milk product samples, with the overall prevalence of 31.5 %. All isolates were genotypically confirmed by PCR-based molecular method by targeting 16S rRNA gene. Out of 63 isolates, 38.33% (n=46) were isolated from raw milk and 21.25% (n=17) were isolated from milk products. District wise highest prevalence was observed in Durg district (40%) followed by Rajnandgaon district (17.00%) and least from Raipur and Bilaspur district (25%).

All 63 *E. coli* isolates were screened for their antibiogram pattern against different antibiotics. Among all 63 isolates, 56 (88.88%) were found to be resistant for Amoxycillin followed by 55 (87.30%) for Amoxyclav, 47 (74.60%) for Cephalexin, 37 (58.73%) for Cefotaxime, 14 (22.22%) for Gentamicin, 13 (20.63%) for Oxytetracycline and 12 (19.04%) for Ciprofloxacin. Out of 63 isolates, 49 (77.77%) were found sensitive to Oxytetracycline and 48 (76.19%) isolates were sensitive to Ciprofloxacin, 29 (46.03%) for Gentamicin and 13 (20.63%) for Cephalexin, 2(6.34%) for Cefotaxime. Whereas, none of the isolates showed sensitivity against Amoxycillin and Amoxyclav. Results further revealed that out of 46 *E. coli* isolates obtained from raw milk, 91.30% were found to be

more resistant against Amoxyclav and from milk products highest resistance (100%) for Amoxycillin was observed in isolates.

Out of 46 *E. coli* isolates from raw milk, 42 (91.30%) isolates showed highest resistance to Amoxyclav, 39 (84.78%) to Amoxycillin, 32 (69.56%) to Cephalexin, 30 (65.21%) to Cefotaxime, 11 (23.91%) to Gentamicin, 10 (21.73%) to Ciprofloxacin, 8 (17.39%) to Oxytetracycline. For milk products, out of 17 isolates, 17 (100%) isolates showed highest resistance to Amoxycillin followed by 15 (88.23%) isolates to Cefalexin, 13 (76.47%) isolates to Amoxyclav, 7 (41.17%) isolates to Cefotaxime, 5 (29.41%) isolates to Oxytetracycline, 3 (17.64 %) isolates to Gentamicin and 2 (11.76 %) isolates to Ciprofloxacin.

Highest multiple antibiotic resistance (MAR) index for *E. coli* isolates was 1 (2 isolate) followed by 0.85 (3 isolates), 0.71 (10 isolates), 0.56 (20 isolates),0.42 (15 isolates), 0.28 (11 isolates), 0.14 (2 isolates). Districtwise in Durg district, out of 20 isolates, 1 isolate showed 0.71 MAR index, 3 isolates showed 0.56, 6isolates showed 0.42, 8 isolates showed 0.28, 2 isolates showed 0.14 MAR index, none of the isolates showed for 0.85 and 1 MAR index. In Raipur district, out of 13 isolates, 2 isolates were resistant to one antibiotic and showed a MAR index of 0.71, 4 isolates showed 0.56, 5 isolates showed 0.42, 2isolates showed 0.28 and none of the isolates showed 0.14, 0.85, 1 MAR index. From Bilaspur district, out of 13 isolates, 3 isolates showed 0.71 MAR index, 7 isolates showed 0.56 MAR index and 3isolates showed 0.42 MAR index and none of the isolates show for 0.14, 0.28, 0.85 and 1 MAR index. In Rajnandgaon district, out of 17 isolates, 2 isolates showed 1 MAR index, followed by 3 isolates showed 0.85, 4 isolates showed 0.71, 6 isolates show 0.56, 1 isolate showed 0.42,

1 isolate showed 0.28 MAR index and none of the isolates showed for 0.14 MAR index.

Out of 63 *E. coli* isolates, 25 (39.68 %) were phenotypically identified as presumptive ESBL producers. To identify β-lactamase-encoding genes $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} multiplex PCR was used. Among 25 *E. coli* isolates 8 (12.69%) isolates were displayed expression of bla_{TEM}, 9 (14.28 %) isolates displayed expression of $bla_{\text{CTX-M}}$ and 8 (12.69 %) isolates were displayed expression of bla_{SHV} gene. In *E. coli* isolates obtained from raw milk products, 7 (15.21%) isolates expressed bla_{TEM} gene, 8 (17.39 %) isolates expressed $bla_{\text{CTX-M}}$ gene and 6 (13.04 %) isolates expressed bla_{SHV} gene. From milk products, 1 (5.88%) isolate expressed bla_{TEM} and $bla_{\text{CTX-M}}$gene, respectively and 2 isolates were harbored bla_{SHV} genes.

E. coli isolates displayed Tetracycline and Fluoroquinolone resistance were molecularly characterized by multiplex PCR method. Among 13 Tetracycline resistant *E. coli* isolates, 7 (53.84%) isolates harbored *tet*A gene whereas only1 (7.69 %) isolate harbored *tet*B gene. Furthermore, among 12 Fluoroquinolone resistant *E. coli* isolates, 4 (33.33%) isolates expressed *gyr*A whereas, 6 (50%) isolates expressed *par*C gene.

CONCLUSION

- A total of 63 *E. coli* isolates were recovered from milk and milk products, with an overall prevalence of 31.5%. The prevalence of 38.33% and 21.25% was observed in raw milk and milk products, respectively.
- Among four districts under study, highest prevalence of *E. coli* was recorded from Durg (40%), followed by Rajnandgaon (34%) and least from Bilaspur (24%) and Raipur (24%).
- *E. coli* isolates were found highly resistant against amoxycillin (88.88%) and highly sensitive against oxytetracycline (77.77%). Highest MAR index of 1 was recorded for only two isolates.
- Among 25 *E. coli* isolates 8 (12.69%) isolates were displayed expression of bla_{TEM}, 9 (14.28 %) isolates displayed expression of $bla_{\text{CTX-M}}$ and 8 (12.69 %) isolates were displayed expression of bla_{SHV} gene.
- Among other resistance genes, the *tet*A gene was most prevalent (53.84%), followed by *par*C gene (50%), *gyr*A gene (33.33%) and *tet*B gene (7.69%).
- There is an urgent need to find alternative antimicrobials that are effective against *E. coli* and at the same time safe for humans due to the high degree of MDR found in field isolates of *E. coli*

SUGGESTION FOR FUTURE RESEARCH WORK

1. Besides, AmpC, β-lactamase- producing *E. coli* may be screened in raw milk and milk products.
2. Other resistance genes *viz tet*C , *tet*D and PMQR genes may be screened in ESBL positive *E. coli* isolates from milk and milk products.
3. More comprehensive study in raw milk and milk products with more number of samples from different districts of Chhattisgarh is required to evaluate the prevalence of *E. coli* in raw milk and their antimicrobial resistance.
4. More antibiotics may be included in future research work.

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THESIS ABSTRACT

Title of Thesis: Antimicrobial resistance and molecular characterization of *Escherichia coli* isolated from milk and milk products in Chhattisgarh

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Milk is a major part of human food and plays a prominent role in the human diet. Milk and milk products contain high moisture and are rich in vitamins and minerals. Hence, its high nutritional value provides the ideal environment for bacterial multiplication. Raw milk and milk products were contaminated under unhygienic conditions. For milk to be safer for consumers it must be reasonably free from microbes, such as *Escherichia coli*. The present study was undertaken to isolate and identify the *E. coli* of public health significance in raw milk and milk products along with their antibiogram pattern. A total of 200 samples (120 raw milk and 80 milk products samples) were collected from Durg, Raipur, Bilaspur, and Rajnandgaon districts of Chhattisgarh and analyzed for the isolation and identification of *E. coli* by conventional cultural technique and further confirmed by biochemical test and molecular techniques. All *E. coli* isolates were also tested for their antibiotic susceptibility pattern by disc diffusion technique against 7 antibiotics. Prevalence of Extended Spectrum Beta-Lactamase (ESBL) genes ($bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}) and other resistance genes (*tet*A, *tet*B, *gyr*A and *par*C) among *E. coli* isolates were also determined.

A total of 63 *E. coli* isolates were recovered from 200 raw milk and milk product samples, with an overall prevalence of 31.5%. All isolates were genotypically confirmed by PCR-based molecular method by targeting 16S rRNA gene. Out of 63 isolates, 38.33 % (n=46) were isolated from raw milk and 21.25 % (n=17) were isolated from milk products. All isolates were found sensitive to

Oxytetracycline and Ciprofloxacin whereas the majority of isolates showed multiple antibiotics resistance. Maximum resistance was observed against Amoxyclav (88.88%) and Amoxycillin (87.30%). The highest Multiple Antibiotic Resistance (MAR) index of 1 was observed for 2 isolates and MAR index for all *E. coli* isolates varied between 0.14 to 1.

All 63 *E. coli* isolates were further screened by phenotypic method for ESBL production and 25 *E. coli* isolates were identified as presumptive ESBL producers. All 25 isolates were found positive either for $bla_{\text{CTX-M}}$, $bla_{\text{SHV}}$and bla_{TEM} genes by molecular technique. Among 25 presumptive ESBL *E. coli* isolates, 8 (12.69 %) isolates harbored bla_{TEM},, 9 (14.28 %) isolates harbored $bla_{\text{CTX-M}}$ gene and 8 (12.69 %) isolates displayed expression for bla_{SHV} gene. Among 13 Tetracycline-resistant isolates, 7 (53.84%) isolates harbored *tet*A gene whereas only 1 (7.69%) isolate harbored *tet*B gene. Among 12 Fluoroquinolone resistant isolates, 4 (33.33%) contained *gyr*A gene whereas 6 (50%) isolates contained *par*C gene. ESBL causes a rapid increase of multidrug-resistant bacteria and also reduces the efficacy of a wide range of β- lactam antibiotics. Antibiotics are regularly added to feed as feed additives in intensive animal production at sub-therapeutic doses to stimulate growth, improve feed efficiency, and avoid infections. The presence of ESBL-producing *E. coli* in raw milk is a serious public health threat. However, all pathogenic microorganisms are destroyed after pasteurization, including resistant bacteria, making milk and other milk products generally safe for human consumption.

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