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Antibiotic resistance profile of *Escherichia coli* isolated from raw milk and raw milk cheese

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Abstract
Raw milk and raw milk cheese were collected from Puducherry and Cuddalore. After collection of samples, in case of raw milk, bacterial Pre-enrichment was performed in 225 ml of 1% buffered peptone water with 25 ml of raw milk sample and in case of raw milk cheese, Pre-enrichment was performed in 225 ml of 1% buffered peptone water with 25 g of raw milk cheese. After Pre-enrichment**,** isolation and identification of the target organism was carried out. The Pre-enriched raw milk and raw milk cheese samples were streaked in EMB agar plate and Mac Conkey agar plates. Then they were incubated at 37 ℃ for 24hrs. *E. coli* was identified by the pink colour colonies in Mac Conkey agar. If EMB agar plate contains dark centered colonies with greenish metallic sheen, they were considered as positive for *E. coli.* The isolates were sub cultured to get pure culture. The isolates were identified based on morphological and biochemical characterization. The antibiotic susceptibility testing was carried out for the isolates against the selected antibiotics Ampicillin, Tetracycline, Kanamycin, Erythromycin, Oxacillin, Streptomycin, Amikacin, Gentamicin, Cephalothin, Ciprofloxacin and Chloramphenicol. Among the ten raw milk isolates and ten raw milk cheese isolates, raw milk isolate RMS 4 showed resistance towards five selected antibiotics. RMS 4 isolate was identified by 16S r RNA sequencing and it was identified with 99.92% of similarity as *E. coli*.

Keywords: Raw milk, raw milk cheese, pre-enrichment, antibiotic susceptibility and 16S r RNA sequencing

Introduction
Escherichia coli is a type of bacteria and it is commonly found in human and animal intestine. *E. coli* is an important bacteria of gut flora (Tahlina Tanzin *et al*., 2016, Eckbung *et al*., 2005) [23]. *E. coli* belongs to the family Enterobacteriaceae and it is a Gram-negative rod-shaped bacterium and non-spore forming. Infections of *E. coli* can spread typically through the consumption of contaminated food. While most *E. coli* strains are harmless, only a few can cause serious illnesses like food poisoning, mild gastroenteritis, severe urinary tract infections, kidney failure, meningitis and even pneumonia. Pathogenic strains of *E. coli* are classified into different groups on the basis of their virulence factor. These strains typically produce toxins that can damage the intestinal lining and cause symptoms such as diarrhea, vomiting, and abdominal pain. (Mojtaba Bonyadian *et al.*, 2014) [17]. The best way to prevent *E. coli* infection is to practice good hygiene, including washing hands frequently and thoroughly cooking food to a safe temperature and avoiding contact with potentially contaminated water or food.

Raw milk
Milk is an excellent medium for the growth of many microorganisms (Tahlina Tanzin *et al*., 2016, Haftay Abraha Tadesse *et al*., 2018) [23, 8]. Contamination of milk with several bacteria occurs during milking process from the milking personnel and utensils used for milking. Through the teat canal microorganisms may enter the udder, bacteria may come out through milk. The pathogen present in milk largely depends on faecal contamination (Tahlina Tanzin *et al*., 2016, Daniela Benevides Melo *et al*., 2015, Segni Bedasa *et al*., 2018) [7, 21, 23]. And this is because of poor sanitation is maintained during the collection and processing of milk from cattle. Raw milk is milk that has not undergone the process of pasteurization, which involves heating the milk to kill harmful bacteria present in it. Raw milk has various health benefits.

It is a rich source of nutrients such as calcium, protein and vitamins. Raw milk is directly obtained from animals such as cows or goats, and it is typically sold as a natural and unprocessed food product. Harmful bacteria in raw milk, such as *E. coli*, *Salmonella*, and *Listeria* can cause serious illnesses in people with weakened immune system (Nobili *et al*., 2016, Anna C.L.P. de Campos *et al.*, 2017, Christopher L baylis *et al*., 2009) [18, 5, 6].

Raw milk cheese

Raw milk cheese is made from unpasteurized milk (Alexandra Tabaran *et al*., 2016, Jordan Madic *et al*., 2011, Paneto *et al*., 2006) [12, 11, 19] and the pasteurization process is not involved in its production. The milk is obtained directly from the farm for specific quality and safety standards. The milk is processed using traditional cheese-making techniques. During cheese making process, the raw milk may be slightly heated to promote coagulation. Raw milk cheese can come in many different varieties from soft and creamy brie to hard and crumbly cheddars. Well-known raw milk cheese include Roquefort cheese from France, Parmigiano Reggiano from Italy. Like raw milk, raw milk cheese also contains harmful bacteria (Abolfazl Jafari-Sales *et al.*, 2019, Marwa E.A. Aly *et al*., 2012, Vernozy-Rozand *et al*., 2005, Miranda *et al.*, 2009) [1, 15, 24, 16]. Raw milk cheese is often favored by cheese enthusiasts for its unique flavor and texture. Because the milk used in the preparation of cheese is not heated to high temperature. It retains wider range of bacteria that contribute to the development of flavor and texture in the cheese. And it is preferred for its taste and texture. It is believed that raw milk cheese has health benefits over pasteurized cheese. Raw milk and cheese are responsible for a disproportionate number of food borne illness outbreaks compared to other products.

Mastitis

Mastitis is one of the common problem in dairy industry affecting cows. Cattles can become infected with *E. coli* in several ways including contaminated feed and exposure to other infected animals or improper hygiene practices during milking. *E. coli* infection in cows can cause mastitis. Mastitis in cattle can have significant impact on milk production and, as well as health and well-being of animal. During mastitis, the cow has inflammation on the mammary gland. It's a major cause of economic loss in dairy industry. Mastitis caused by *E. coli* is typically associated due to environmental condition or poor hygiene such as poor ventilation or wet bedding. There are several different types of mastitis, including clinical mastitis and subclinical mastitis. Clinical signs of *E. coli* infection in cows include swelling, redness in udder as well as decrease in milk production (Hassan Momtaz *et al*., 2012) [9] which can also affect the quality of milk. Those milk from affected area may also be abnormal in color, consistency or smell. Subclinical mastitis, on the other hand, does not typically show visible signs of inflammation but can lead to decreased production.

Antibiotic resistance

Antibiotic resistance is the ability of bacteria to resist the antibiotic effects. Its means the used drugs become completely less effective or ineffective in treating bacterial infections. This was occurred when antibiotics are used in animal and agriculture to promote growth or prevent disease. Overuse and misuse of antibiotics is the primary reason behind the development of antibiotic resistance (Mojtaba Bonyadian *et al*., 2014, Kyeonga Jang *et al*., 2018, Hugo Figueiredo Botelho Damaceno *et al*., 2015, Ana Belen Florez & Baltasar Mayo, 2015) [17, 13, 10, 3]. When antibiotics are used too frequently or inappropriately, they kill off the susceptible bacteria and leave behind only the resistant ones which can then multiply and spread. It is also important for the development of new antibiotics and alternative therapies to treat infections. The presence of antibiotic resistant *E. coli* in raw milk and raw milk cheese can pose a significant health risk to the consumer (KálmánImre *et al*., 2022, Marek Vrabec *et al*., 2015) [12, 14]. Consumption of these products can lead to transmission of antibiotic-resistant bacteria and it increase the risk of antibiotic-resistant bacterial infection (Souhir Badi *et al*., 2018, Shlomo E Blum *et al*., 2013, Adrian Canizalez-Roman *et al*., 2013) [22, 2]. The present study is focused to find out the antibiotic resistance pattern of *E. coli* isolated from raw milk and raw milk cheese and their identification.

Materials and Methods

Collection of samples

In this study raw milk and raw milk cheese samples (Figure 1) were collected from various places around Cuddalore and Puducherry and they were analyzed for detection of *E. coli* strains.

Pre-enrichment

After collection of samples, in case of raw milk, bacterial pre-enrichment was performed in 225 ml of 1% buffered peptone water with 25 ml of raw milk sample and in case of raw milk cheese, pre-enrichment was performed in 225 ml of 1% buffered peptone water with 25g of raw milk cheese (Figure-2).

Isolation of organism

After Pre-enrichment**,** isolation and identification of the target organism was carried out. The pre enriched raw milk and raw milk cheese samples were streaked onto EMB agar (HI Media) plate and Mac Conkey agar (HI Media) plates. Then they were incubated at 37 ℃ for 24hrs. *E coli* was identified by the pink colour colonies in MacConkey agar, If EMB agar plate contains dark centered colonies with greenish metallic sheen, they were considered as positive for *E. coli.* The isolates were sub cultured to get pure culture. The isolates were identified based on morphological, biochemical and molecular characterization.

Identification of bacterial isolates

Morphological and biochemical characterization of bacterial isolates

The bacterial isolates were identified based on morphological features such as Gram staining and motility test. Isolates were subjected to biochemical test such as Indole, Methyl red, Voges Proskauer, Citrate utilization, Catalase test, Oxidase test and TSI test. Molecular characterization of the potent antibiotic resistant isolate was identified by 16S rRNA sequencing.

Antibiotic susceptibility testing

The antibiotic susceptibility testing was carried out by Kirby-Bauer disc diffusion method using Muller-Hinton agar (HI Media). The antibiotic discs were used with the

isolates to identify the antibiotic resistance of it. Antibiotic discs (HI Media) used were Gentamicin (GEN) (10 mcg), Ciprofloxacin (CIP) (5 mcg), Amikacin (AK) (30 mcg), Streptomycin (S) (10 mcg), Cephalothin (CEP) (30 mcg). Kanamycin (K) (30 mcg), Chloramphenicol (C) (50 mcg), Ampicillin (AMP) (10 mcg), Erythromycin (E) (15 mcg), Oxacillin (OX) (1 mcg) and Tetracycline (TE) (30 mcg). Then the zone of inhibition was measured and compared with the interpretation chart to determine the resistance of *E. coli.* The isolates were inoculated in tryptic soy broth (HI Media) and incubated at 37 °C for 6 hours and observed for visible turbidity. Then the sterile petriplate were plated with Muller Hinton Agar (HI Media) and were allowed to solidify. The bacterial cultures were swabbed over agar surface aseptically. Then gently placed the antibiotic discs on the agar surface at equal distance and were incubated for 24 hours at 37 °C. After incubation the zone of inhibition was measured in mm. Then the zone of inhibition is compared with the interpretation chart to determine the resistance of *E. coli* isolates.

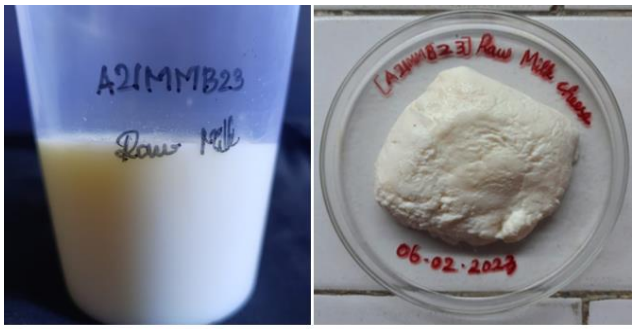
Results and Discussion

Identification of bacterial isolates from raw milk and raw milk cheese

The morphological, biochemical and cultural characteristics of the isolated strains from raw milk and raw milk cheese were studied. Raw milk and Raw milk cheese isolates were screened for *E coli* using specific media EMB agar and differential media MacConkey agar (Figure-3). Isolates were identified by morphological (Gram negative motile rods), biochemical (Indole positive, Methyl Red positive, VP negative, Citrate negative, Catalase positive, Oxidase negative, Urease negative, Acid butt, acid slant and gas roduduction in Triple sugar Iron Agar), and molecular characterization using 16S rRNA sequencing technique. Blast analysis was carried out and the isolate was confirmed as *E coli* with similarity percentage of 99.92% (Figure-5).

Antibacterial susceptibility testing

Antibiotic susceptibility testing was carried out for the isolates of raw milk and raw milk cheese. Different antibiotic disc were used such as Ampicillin (Amp), Tetracycline (TE), Gentamicin (GEN), Streptomycin(S), Cephalothin (CEP), Kanamycin (K), Amikacin (AK), Ciprofloxacin (CIP), Chloremphenicol (C), Erythromycin (E) and Oxacillin (OX). Resistant pattern of isolates towards the antibiotics were studied. (Table-1 & Figure-4). From those isolates, RMS 4 showed resistance towards five antibiotics which was identified by 16S r RNA sequencing.

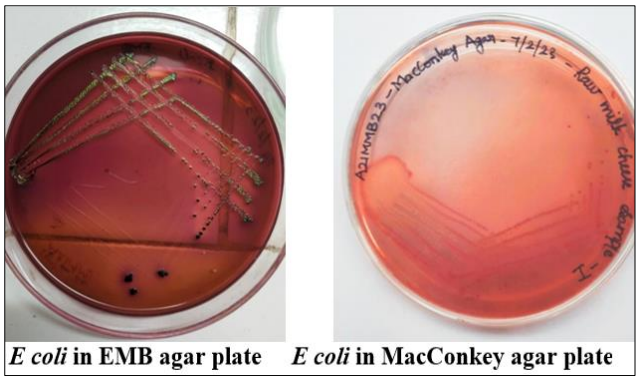


Raw Milk **Raw Milk Cheese**

Fig 1: Showing raw milk and raw milk cheese samples

Pre enrichment for raw milk sample **Pre enrichment for raw milk cheese**

Fig 2: Showing pre-enrichment of raw milk and raw milk cheese samples



***E coli* in EMB agar plate** ***E coli* in MacConkey agar plate**

Fig 3: Showing *E. coli* in EMB agar and mac Conkey agar isolated from raw milk

Table 1: Antibiotic susceptibility testing of bacterial isolates from raw milk

S. No	Antibiotics	Isolates									
		RMS1		RMS 2		RMS 3		RMS 4		RMS 5	
		Zone size in mm	Susceptibility	Zone size in mm	Susceptibility	Zone size in mm	Susceptibility	Zone size in mm	Susceptibility	Zone size in mm	Susceptibility
1	Gentamycin (GEN)	26	S	18	S	16	S	17	S	18	S
2	Ciprofloxacin (CIP)	30	S	40	S	24	I	21	**R**	33	S
3	Chloremphenicol (C)	12	R	30	S	23	S	21	S	25	S
4	Streptomycin (S)	24	S	11	R	19	S	13	S	20	S
5	Cephalothin (CEP)	18	S	21	S	17	I	14	**R**	20	S
6	Ampicillin (Amp)	-	R	16	I	-	R	-	**R**	14	I
7	Erythromycin (E)	14	I	15	I	17	I	9	**R**	15	I
8	Oxacillin (OX)	-	R	-	R		-	R	**R**	-	-
9	Amikacin (AK)	21	S	20	S	20	S	22	S	18	S
10	Tetracycline (TE)	18	S	17	S	15	S	18	S	20	S
11	Kanamycin (K)	17	I	15	I	18	S	17	I	13	R

R-Resistance, I-Intermediate, S-Sensitive

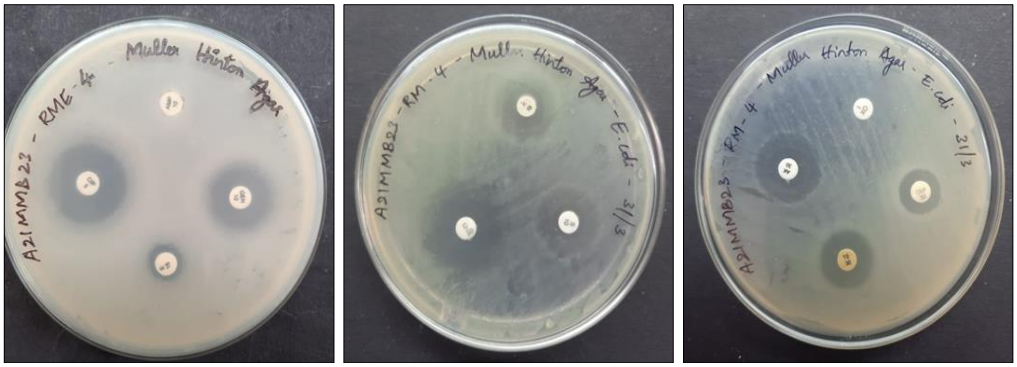


Fig 4: Showing Antibiotic susceptibility testing of Isolate RMS 4 using antibiotics

Tetracycline (TE), Ampicillin (AMP), Gentamicin (GEN), Cephalothin (CEP), Ciprofloxacin (CIP), Amikacin (AK), Kanamycin (K), Oxacillin (OX), Chloremphenicol (C), Streptomycin (S), Erythromycin (E).

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Escherichia coli strain BE42 16S ribosomal RNA gene, partial sequence	2383	2383	99%	0.0	99.92%	EF560785.1
Escherichia coli strain GYX208DH4E-2 chromosome, complete genome	2381	16659	100%	0.0	99.85%	CP104851.1
Escherichia coli strain EC0430 chromosome, complete genome	2381	16365	100%	0.0	99.85%	CP123046.1
Escherichia coli strain E22 chromosome, complete genome	2381	16670	100%	0.0	99.85%	CP123036.1
Escherichia coli strain 20-20 chromosome, complete genome	2381	16670	100%	0.0	99.85%	CP123029.1
Escherichia coli strain 20-16 chromosome, complete genome	2381	16670	100%	0.0	99.85%	CP123024.1
Escherichia coli strain 18-4 chromosome, complete genome	2381	16670	100%	0.0	99.85%	CP123013.1
Escherichia coli strain 19-7 chromosome, complete genome	2381	16670	100%	0.0	99.85%	CP123017.1
Shigella flexneri strain HNJZ 16S ribosomal RNA gene, partial sequence	2381	2381	100%	0.0	99.85%	OQ771960.1
Escherichia coli VE-V10-F DNA, complete genome	2381	16605	100%	0.0	99.85%	AP027971.1
Escherichia coli strain RHB44-C13 chromosome, complete genome	2381	16570	100%	0.0	99.85%	CP099051.1
Escherichia coli strain RHB44-C11 chromosome, complete genome	2381	16575	100%	0.0	99.85%	CP099056.1
Escherichia coli strain RHB25-E3-C05 chromosome, complete genome	2381	16481	100%	0.0	99.85%	CP099167.1
Escherichia coli strain RHB20-E3-C03 chromosome, complete genome	2381	16603	100%	0.0	99.85%	CP099203.1
Escherichia coli strain RHB03-E1-C04 chromosome, complete genome	2381	16609	100%	0.0	99.85%	CP099287.1
Escherichia coli strain RHB47-SO-C08 chromosome, complete genome	2381	16592	100%	0.0	99.85%	CP098927.1
Escherichia coli strain RHB43-SE-C07 chromosome, complete genome	2381	16570	100%	0.0	99.85%	CP099071.1
Escherichia coli strain RHB28-SO-C04 chromosome, complete genome	2381	16609	100%	0.0	99.85%	CP099142.1
Escherichia coli strain RHB28-SO-C01 chromosome, complete genome	2381	16631	100%	0.0	99.85%	CP099147.1
Escherichia coli strain RHB25-E3-C03 chromosome, complete genome	2381	16481	100%	0.0	99.85%	CP099168.1
Escherichia coli strain RHB20-E3-C04 chromosome, complete genome	2381	16603	100%	0.0	99.85%	CP099201.1
Escherichia coli strain RHB11-E4-C04 chromosome, complete	2381	16598	100%	0.0	99.85%	CP099243.1

Fig 5: Blast analysis and identification of the strain RMS 4 as *E. coli*

Mojtaba Bonyadian *et al.*, (2014) [17] isolated the enterotoxigenic and enteroaggregative *E. coli* isolates from raw milk and unpasteurized cheese by streaking the diluted sample onto the Mac Conkey agar plates, and after incubation of 24 hrs red color colonies were observed and performed morphological and biochemical tests using the isolated colonies.

In the present study, the isolation of *E. coli* from raw milk and raw milk cheese were studied by performing pre enrichment for both raw milk and raw milk cheese sample with buffered peptone broth and after incubation, they were streaked onto specific media EMB agar and MacConkey agar. After 24 hours of incubation, pink colonies from Mac Conkey agar and dark entered with greenish metallic sheen colonies in EMB agar were isolated. Morphological and biochemical characterization of the isolates were done.

Reza Ranjbar *et al.*, (2018) performed antibiotic resistance studies for Shiga toxin producing *Escherichia coli* by disk diffusion method. Muller-Hinton Agar media was used for antibiotic susceptibility test. Susceptibility of *E. coli* isolates were tested against antibiotic disks like tetracycline, ampicillin, cefotaxime, gentamicin (10 µg/disk), cipro-floxacin, amikacin, ceftazidime, imipenem, cotrimoxazole, enrofloxacin, sulfamethoxazole, trimethoprim, streptomycin and chloramphenicol. The inoculated plates were incubated and results were interpreted.

In the present study, antibiotic resistance for *E. coli* isolates from raw milk and raw milk cheese were found out by using Kirby-Bauer disk diffusion method. The cultures were inoculated on tryptic soy broth. After incubation the culture were swabbed on to the Muller-Hinton Agar plates. Eleven antibiotic disks were placed on the plates. After incubation, the zone of inhibition was measured and interpreted. Resistant pattern of isolates towards the antibiotics were studied. From those isolates, RMS-4 showed resistance towards five antibiotics which was identified by 16S r RNA sequencing.

Reza Ranjbar *et al.*, (2018) [20] studied that the bacterial isolates were identified by 16S rRNA-based PCR. Bacterial strains were sub-cultured in Luria-Bertani broth and incubated for 48hrs. Genomic DNA was extracted using a DNA extraction kit. quality and concentration were assessed. The DNA extract was amplified using specific primers and PCR reagents. The amplification was performed in a thermal cycler with a protocol consisting of denaturation, annealing, and extension steps. This method allows for the detection of bacterial colonies based on their 16S rRNA sequences.

Molecular characterization in this study for RMS 4 was carried out by 16S rRNA sequencing. The PCR was carried out by performing amplification, denaturation and annealing and it was identified as *E. coli* with 99.92% similarity was found out and the phylogenetic tree was constructed. The isolate was submitted in the GEN bank with the accession number OR244368.

Conclusion

Emergence and spreading of antibiotic resistance in *E coli* strains screened from raw milk and raw milk cheese denotes the drug resistance threat to human beings who used to consume these milk and milk products. The results indicates the need for effective sanitary measures to improve the food safety because the presence of *E coli* in ready to eat dairy products suggests possible fecal contamination and lack of hygiene.

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Competing interests

Authors have declared that no competing interest exist.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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