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Prevalence of *Escherichia coli* in Raw Cow's Milk in Cuddalore District

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A B S T R A C T

Milk is considered a complete and nutritious food for the new-born mammal and human beings, but it is considered as a good medium for many microorganisms. The consumption of raw milk and its products, which lead to the transmission of various diseases. The ability of raw milk to support the growth of several pathogenic microorganisms that can lead to spoilage of the product and infections in consumers. In the present investigation a survey was undertaken to collect raw milk samples from Local Vendorsin cuddalore district. Based on the Morphological and Biochemical characterization presence of *E.coli* were confirmed. Out of Twelve milk sample tested, the sample 2 sample 4, sample 7 and sample 9 exhibit the presence of *E.coli*. So it is observed raw milk attributed from different dairy farm is enabling cross contamination resembling growth of various microbial organism by different ways such as they are mixed fresh clean milk with mastitis milk, unclean hands of workers, unclean utensils and unhygienic water supply for washing the utensils could be the source for accelerating the bacterial contamination.

Introduction

Raw untreated milk is still used by large number of farm families and workers and by a growing segment of the general population who believe that the milk is not only safe but also imparts beneficial health effects that are destroyed by pasteurization (LeJeune and Rajala-Schultz, 2009). For this reason, utilization of both raw untreated milk and raw milk cheeses has frequently been associated with food-borne illness. Especially, developing countries are mostly affected by food-borne infections (Carbas *et al.*, 2012; Bedasa *et al.*, 2018) because of the prevailing poor food handling and sanitation practices, inadequate food safety regulatory systems, lack of financial resources to invest in safer equipment, and lack of education for food-handlers (FAO and WHO, 2004; Oliver, 2005).

Numerous epidemiological reports have implicated raw milk is usually colonized by a variety of zoonotic foodborne pathogens such as

Campylobacter jejuni, enterohaemorragic Escherichia coli, Salmonella typhimurium, Listeria monocytogenes, Staphylococcus aureus and *Yersinia enterocolitica*. These pathogens have been originated from environment in the farm, mixing clean milk with mastitis milk, manure, soil, and contaminated water (Gwida and EL-Gohary, 2013; Nigatu *et al.,* 2017).

Among the major infectious agents, *E. coli* has been associated with milk and some of dairy products (Abebe *et al.,* 2014; Bedasa *et al.,* 2018). *E. coli* is Gram-negative, facultative anaerobic, rod-shaped and highly motile bacteria that belong to the family Enterobacteriaceae, and a normal inhabitant of the intestines of animals and humans (Tchaptchet and Hansen, 2011; Virpari *et al.,* 2013; Asmelash, 2015) but its recovery from food may be of public health concern due to the possible presence of enteropathogenic and/or toxigenic strains which lead to wide variety of enteric and extraintestinal diseases in animals (Fairbrother *et al.,* 2002; Asmelash, 2015).

E. coli have several types of strains that are divided into six groups of pathotypes based on the mechanism of disease cause. Enteropathogenic *E. coli* (EPEC), Attaching and effacing *E. coli* (A/EEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), EHEC and Enteroaggregative *E. coli* (EAEC). *E. coli* strains that produce the Stx toxins have been referred to as Vero Toxin-producing *E. coli* (VTEC), Shiga-toxigenic E. coli (STEC) and enterohaemorrhagic *E. coli* (EHEC) (Karmali, 1989; Nataro and Kaper, 1998; Asmelash, 2015; Saba *et al.,* 2015).

E. coli O157: H7 are toxigenic strains that cause life threating syndromes and resulted in an estimated 74,000 cases and 61 deaths annually in USA as a result of outbreaks arising from consumption of contaminated cattle products, especially meat and raw milk (Bedasa *et al.,* 2018). Later, outbreaks were traced to other dairy products such as yogurt and cheese (Doyle *et al.,* 2006; Mora *et al.,* 2007). *Escherichia coli O15 7:H7* has also been found in the intestines of healthy cattle, deer, goats, and sheep. However, cattle have been identified as a major reservoir of *E. coli O157: H7* and consumption of foods of cattle origin such as beef and dairy products have been associated with some of the largest food poisoning outbreak s in which this organism was identified as the etiologic agent (Acha and Szyfress, 2001; Rey *et al.,* 2003; Perelle *et al.,* 2007).

Detection of *E. coli O157: H7* is dependent on distinguishing the pathogenic serotypes from normal fecal flora containing commensal strains of *E. coli* (Battisti *et al.,* 2006; Woynshet, 2014). Fortunately, *E. coli O157: H7* has two unusual biochemical markers; delayed fermentation of D-sorbitol and lack of β-D-glucuronidase activity, which help to phenotypically separate *E. coli* O157: H7 isolates from nonpathogenic *E. coli* strains. One of these markers (delayed sorbitol fermentation) enables to develop of several selective media (Sorbitol-MacConkey) which aid in the initial recognition of suspicious colonies isolated from bloody stools (Woynshet, 2014). Detection of *E. coli O157: H7* from food samples requires enrichment and isolation with selective and/or indicator media, but lacks specificity to identify STEC (Ji-Yeon *et al.,* 2005).

Therefore the present has been carried in this field (Food Microbiology). Hence, this work was done in the manner of studying the occurrence of *E. coli* in raw milk samples through a series of biochemical tests.

Materials and Methods

Milk Sample Collection

Raw milk samples were collected from lactating of dairy cows. During sampling, the sample was collected aseptically and put in to sterile screw capped bottle and kept in an ice box containing ice packs and taken immediately to microbiology laboratory for bacteriological analysis. Then the sample was stored over night in refrigerator at 4°C and processed within 24hr of sampling. Isolation

and identification of bacteria was done according to the techniques recommended by Quinn *et al.,* 2002.

Isolation and Identification of *Escherichia coli*

For the isolation and identification of *E. coli*, one ml from incubated BPW was transferred to Eosin methylene blue (EMB) (Oxoid) agar and incubated at 37°C for 24 hrs. Morphologically typical colonies producing metallic sheen were taken sorbitol MacConkey agar base (Oxoid) and incubated at 37°C for 24 hr. The purified colonies were then streaked onto nutrient broth and incubated at 37°C for 18-24 hrs for further identification.

Simultaneously another single colony with similar characteristics was picked from agar plate and stained with Gram's stain. The isolate was examined for stain and morphological characteristics using bright-field microscopy. KOH test was then employed to confirm the Gram's reaction (Quinn *et al.,* 2004). Suspected colonies of *E. coli* (pinkish color appearance on MacConkey agar and green metallic sheen on Eosin Methylene Blue was then sub-cultured onto nutrient agar to appreciate colony characteristics and then pure colonies taken from EMB was inoculated on nutrient agar (non-selective media). The isolated colonies were subjected to series of different biochemical tests.

Biochemical Characteristics

The isolated strains were subjected to a series of different biochemical tests using the procedure referred from Bergeys manual of systematic bacteriology to confirm *E. coli* isolates. Catalase test, indole production test, Methyl red test, MR-VP medium, utilization test performed on all suspected isolates to confirm the *E. coli*.

Indole Test

At first peptone broth or SIM media in test tubes is prepared then the tubes were autoclaved at 15 lbs pressure for 15 mins. Using sterile wire, broth is inoculated with the given samples of organism then label the tubes with name of organism. Then the tube is incubated at 37°C for 24-48 hours. After proper incubation, we have added 4-8 drops of kovac's reagent to the tube touching the wall of glass tube Roll each tube between your palms to mix the reagent through the culture. Let stand for a while and observe for the development of cherry red color at the surface of media.

Catalase test: Tube Method

At first1-2 ml of hydrogen peroxide solution is poured into a test tube. Using a sterile wooden stick or a glass rod, several colonies were observed after18 to 24 hours test organism then after it is immersed in the hydrogen peroxide solution. Then after observe for immediate bubbling

Methyl Red (MR) Test

Prior to inoculation, allow medium to equilibrate to room temperature. Organisms is taken from an 18-24 hour pure culture, then it is inoculated into the medium. Incubate aerobically at 37 degrees Celsius for 24 hours. Following after 24 hours of incubation, aliquots of 1ml of the broth to a clean test tube. Then remaining broth is reincubated for an additional 24 hours. Then after add 2 to 3 drops of methyl red indicator to aliquots andthen Observe for red color immediately

Voges–Proskauer (VP) Test

Prior to inoculation, allow medium to equilibrate to room temperature. Organisms is taken from an 18-24 hour pure culture, lightly inoculate the medium. Then after it is Incubated aerobically at 37 degrees Celsius for 24 hours. After following 24 hours of incubation, aliquot 2 ml of the broth to a clean test tube is Re-incubated and the remaining broth is kept for additional 24 hours. Then after 6 drops of 5% alpha-naphthol is added and mixed well to aerate. Then after 2 drops of 40% potassium hydroxide is added and mix well to aerate then after pink-red color at the surface within 30 min is observed then tube was vigorously shaken for 30-min period.

Citrate Utilization Test

Preparation of the media

In a beaker, 24.28 grams of the dehydrated powder or lab-prepared media is added to 1000 milliliters of pure distilled or deionized water. The solution is then heated to bring it to a boil in order to dissolve the medium completely. The dissolved medium is then dispensed into tubes and sterilized in an autoclave at 15 lbs pressure (121°C) for 15 minutes. Once the autoclaving process is complete, the tubes are taken out and cooled at a slanted position to a temperature of about 40-45°C. The position should be maintained in order to obtain butts of 1.5 – 2.0 cm depth.

Utilization test

A well-isolated colony is taken from an 18-24 hour culture with a sterile inoculating needle. The citrate agar tubes are inoculated by streaking the surface of the slant.

The slant should be streaked back and forth with the loop or the inoculating stick. The cap of the test tubes should be left loosened to ensure adequate aeration. The tubes are then incubated aerobically at 35-37°C for up to 4 days. The test tubes should be examined daily for 4 days before discarding the result as a negative. The change in color, if present, is observed.

Results and Discussion

E.coli Identification and Characterization

The results of the present study were found to be positive for E. coli. Isolates were characterized as bright pink color on MacConkey agar plates and showed blue-greenish metallic sheen on EMB agar plate Upon Gram's staining of the isolates under 100x using light microscope, pink-colored, small rod-shaped organisms arranged in single, pairs or short-chain was identified

Biochemical characterization of *E.coli*

The biochemical characteristics of *E. coli* isolate showed positive for catalase, Methyl red and indole test but negative for Voges-Proskauer, urease, and citrate. In addition, reactions in TSI agar slant revealed yellow but with gas and production of hydrogen sulfide was observed. Almost all the isolates of *E. coli* fermented lactose, sucrose and glucose with the production of both acid and gas.

In current study, a total of raw milk samples were collected and processed bacteriologically and biochemical tests were performed to detect *Escherichia coli* from raw milk. All the *E. coli* isolates were able to produce bright pink colored colonies on MacConkey agar, characteristic metallic sheen colonies on the EMB agar and pink colored, small rod-shaped Gram-negative bacilli on Gram's staining. The results of catalase, MR and indole test of the *E. coli* isolates were positive but the V-P test was negative which are in agreement with the reports of (Zinnah *et al.*, 2007).

The pattern of sugar fermentation reaction by the isolated *E. coli* with three sugars was observed and produced acid and gas. The isolates were able to ferment glucose, lactose, and sucrose completely. Acid production was indicated by the color change from reddish to yellow and the gas production was noted by the appearance of gas bubbles in the test tubes. This result was in agreement with the findings (Asmelash, 2015; Bedassa, 2018; Zinnash, 2007; Giwida and Gohary, 2013). This result was partially in agreement with the findings of (Beutin *et al.*, 1993 and Sandhu *et al.*, 1996). They reported that although *E. coli* ferments all three basic sugars but it partially ferments sucrose and glucose. Variation of the results may be due to genetic factors and the nature of the inhabitant of the organisms.

Table.1 Total Number of Microbial *E.Coli* Colonies

S.No.	Sample	Total Number of Colonies (10^6 Cfu/ml)
1	Milk Sample 1	2.1±0.01
2	Milk Sample 2	2.9±0.02
3	Milk Sample 3	1.9±0.01
4	Milk Sample 4	2.3±0.01
5	Milk Sample 5	1.1±0.01
6	Milk Sample 6	3.1±0.01
7	Milk Sample 7	3.1±0.01
8	Milk Sample 8	1.7±0.01
9	Milk Sample 9	3.1±0.01
10	Milk Sample 10	1.3±0.01
11	Milk Sample 11	2,4±0.01
12	Milk Sample 12	2.8±0.01

Table.2 Morphological Characteristics of Isolated *E.coli* bacteria

S.No	No.of *E.coli* Isolates	Gram Staining	Motility	Spore formation	Gas
1	Milk Sample 2	Negative	Motile	Non Sporing	+
2	Milk Sample 4	Negative	Motile	Non Sporing	+
3	Milk Sample 5	Negative	Non Motile	Sporing	-
4	Milk Sample 7	Negative	Motile	Non Sporing	+
5	Milk Sample 8	Negative	Non Motile	Sporing	-
6	Milk Sample 9	Negative	Motile	Non Sporing	+
7	Milk Sample 11	Negative	Non Motile	Sporing	-
8	Milk Sample 12	Negative	Non Motile	Sporing	-

Table.3 Biochemical Test Result of Milk Sample

S.No	Sample	Indole	Catalase	Citrate	MR	VP
1	Milk Sample 2	+	+	-	+	-
2	Milk Sample 4	+	+	-	+	-
3	Milk Sample 5	+	+	+	+	-
4	Milk Sample 7	+	+	_	+	_
5	Milk Sample 8	+	+	-	+	_
6	Milk Sample 9	+	+	-	+	-
7	Milk Sample 11	+	-	+	-	-
8	Milk Sample 12	+	_	+	+	-

So, it is observed that from 8 predicted *E.coli* milk sample, we got only 4 pure E.coli positive milk sample. Those milk sample are Milk Sample 2,Milk Sample 4,Milk Sample 7 And Milk Sample 9

Fig.1 Nutrient Culture

Fig.2 Presumptive Test

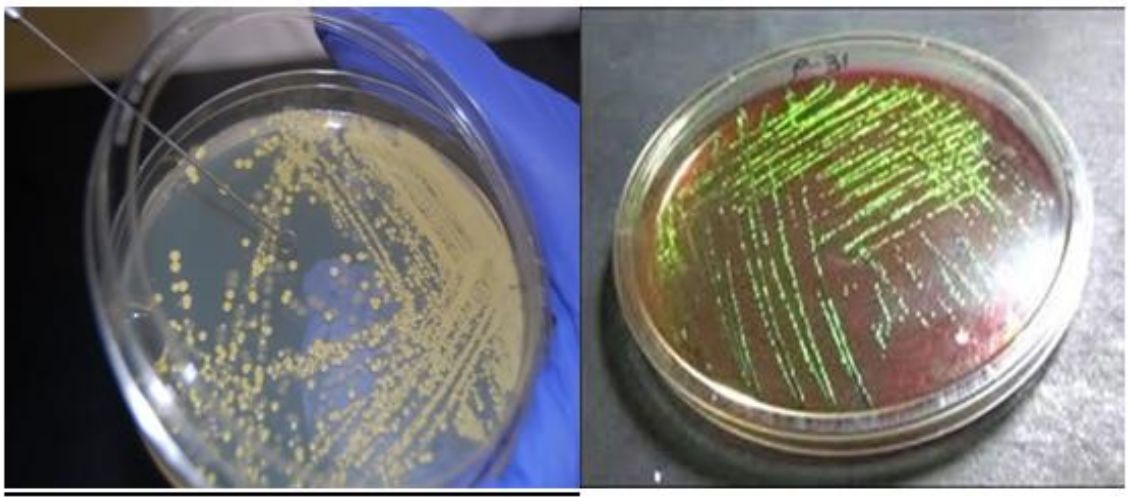


Fig.3 Microscopic View of *E.Coli*

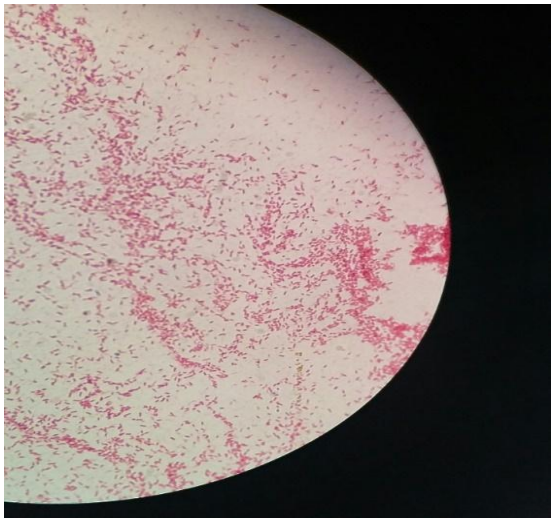
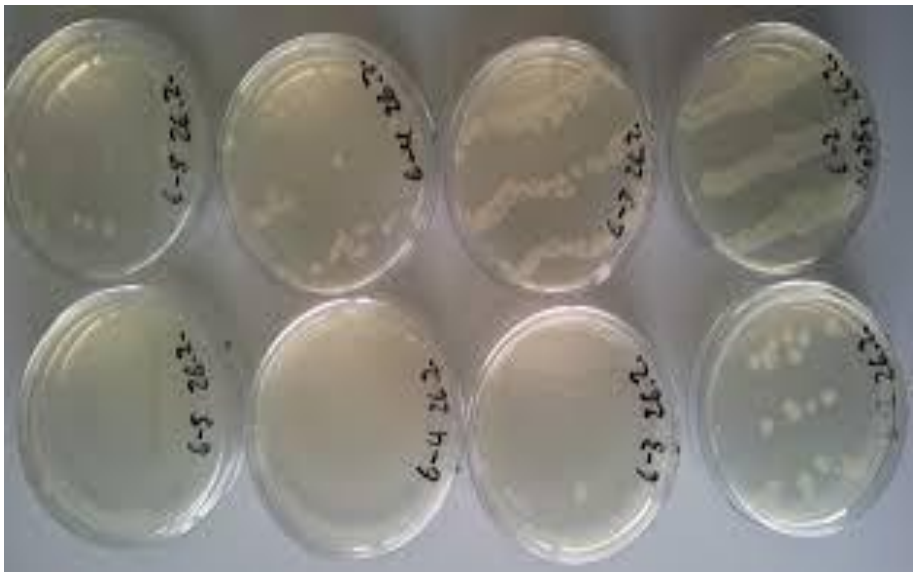


Fig.4 Predicted 8 Coli Sample from that we Obtained 4 Pure *E.coli* Culture



In this study, 42% of the raw milk were contaminated with *E. coli*. In addition, three factors on the farm level were assessed as probable variables related to the higher frequency of samples positive for *E.coli*. There were statistically significant (P< 0.05) associations between body condition, age and breeds of the animals with positive isolates. This finding was comparable with the finding of Iqbal *et al.*, (2004) (40.7%). However, it is much higher than the finding of (Biruke and Shimeles, 2015) (18.6%). This prevalence of *Escherichia coli* is presumably due to the fact that *E. coli* is the commonest environmental contaminants, which is closely associated with hygiene condition of the animals as well as the environment. It becomes pathogenic whenever the hygienic conditions of the animal or environment become poor. Moreover, the existence of high concentration of *E. coli* in milk also indicates the relatively poor quality of milk, related with substandard hygiene of the farm management, milk collection and processing system. The isolation of *E. coli* is of public health significance as this bacterium is known to cause serious gastrointestinal disorders in both young and adult humans (FAO and WHO, 2004).Concerning the type of examined milk samples, the high prevalence of *E. coli* in raw milk may be attributed in padma dairy farm is since milk is mainly transported directly to the dairy plant for processing meanwhile market milk is usually collected from small farms or farmers therefore it will be liable to cross contamination by different ways as mixed fresh clean milk with mastitis milk, unclean hands of workers, unclean utensils and unhygienic water supply for washing the utensils could be the source for accelerating the bacterial contamination. This idea agreed with conditions for contamination of raw milk at different critical points due to less hygienic practices (Reta *et al.*, 2016; Gwida and EL-Gohary, 2013).

The present study was conducted on isolation and identification of *E.coli* from dairy cow raw milk in cuddalore from December to February 2021 and the prevalence of *E.coli* from the collected raw milk was 40%. This indicates that *E.coli* is one of the major problems of dairy cows in milk production that reduced the quality of milk. The distribution of this bacterial pathogen in the herd indicates the economic impact of the disease. Besides, the disease has economic importance and it also do harm the health and well being of human being. In conclusion, the results of the present study provided that microbial quality and safety of raw milk was unsatisfactory. These findings stress the need for an integrated control of *E. coli* from farm production on

to consumption of food of animal origin. In light of the above conclusive remarks, the following recommendations are forwarded:-

Awareness should be created on milk handling practice, storage and milking process to Dairy farmer and milk collectors.

The professionals should apply different methods for prevention and control of the disease.

The professionals should inform the public about the relevance of milk pasteurization before consumption to avoid food born infection

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