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Microbial Hazard Analysis of Branded and Retail Milk Sold in Parbhani City of Maharashtra State, India

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ABSTRACT

An experiment was conducted to study the microbial hazard analysis of milk sold in Parbhani city. A total of 306 milk sample comprising of 34 milk samples from each of five pasteurized milk brands (A, B, C, D and E) and 136 raw milk samples were analyzed during the study. A significantly high (p<0.05) Total Viable Count (TVC) was observed amongst all the sources of milk samples. The raw milk sample were having high TVC (14.99 ± 0.79) than pasteurized milk (14.54) samples. Amongst pasteurized milk samples TVC was high in brand C than other brands. The E.coli counts were significantly high (p<0.05) amongst all the sources. However, E.coli counts of raw milk were the highest (15.05 ± 0.19). A total of 69 E.coli isolates were obtained from 198 milk samples. The identification of these isolates was made by biochemical characterization. A total of 16 E.coli isolates were characterized by using PCR. The isolates have been submitted to Veterinary Type Culture Center (NRCE), Hissar (Haryana) for further typing and maintenance at Repository. Streptococci were also isolated from all the sources. However, a non significant effect of different sources upon streptococcal counts was seen. A total of 70 streptococcal isolations were confirmed with 35.35 percent positivity. An attempt to isolate Salmonella was done but organisms could not be isolated. A total of six clostridial isolates were obtained amongst 198 milk samples. Biochemical characterization has been done. The overall prevalence of 3.03 percent was observed.

Key words: *Microbial hazard, Analysis, Branded and Retail milk, Parbhani city.*

INTRODUCTION

Milk is an essential nutritional food for infants and adults and considered as wholesome diet as it contains all the necessary ingredients required for the infant in the growing stage. Hence milk and milk products are occupying a larger portion of daily food item of modern civilized nation; certainly due to their great food values and palatability. For the provision of milk, many centuries ago man learned to domesticate species of animals for his own consumption.

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In 1980, the Government of India launched the "Operation Flood", a massive program to make the country self sufficient in terms of the demand for milk through co-operative sector in the rural areas, as India was reeling under the shortage of milk and the dairy farmers have contributed significantly in making India number one milk producer in the world. After successful implementation of three phases of 'operation flood', the Food and Agricultural Organization declared India as the leading producer of milk pushing the till then leader USA to second spot. In spite of this success story the per capita consumption of milk is 220 g whereas Indian Council of Medical Research has recommended a minimum consumption criterion of 250 g of milk per head per day. This indicates the shortage of milk for common man and to overcome this shortage and to fulfill the demand of volume of milk, adulteration practices are followed at high rate in the country. Besides the milk supply in the country, especially in the loose market is functioning well, majority of volume of milk comes from rural areas where small farmers keep one or two milking cow or buffaloes without enough hygienic precautions during different stages of milk production. The basic infrastructure required for maintenance of cold chain right from collection till processing of milk is also lacking in the country. Improper handling, storage, use of unhygienic equipments, contaminated water, and infected animal may also contribute to the bacterial contamination of milk.

As milk is a highly nutritious food, ideal for microbial growth and the fresh milk easily deteriorates to become unsuitable for processing and human consumption[9]. High bacterial counts in the milk are indicators of poor hygiene and for pasteurized milk are sign of ineffective pasteurization of milk. Raw milk may contain over 2,000,000 cfu/ml organisms before processing and often contain microorganisms which may cause food borne diseases such as dysentery, salmonellosis, undulant fever, tuberculosis etc.[1]. Microorganisms underscore the importance of milk as vehicle of human infection. For these reasons, the presence of human pathogens in unpasteurized milk remains a public health hazard.

Pathogens that have been involved in food borne outbreaks associated with the consumption of milk include *Escherichia coli & Streptococcus* spp. The presence of these pathogenic bacteria in milk emerged as a major public health concerns[17]. Presence of food borne pathogens in milk is due to direct contact with contaminated sources in the dairy farm, plant environment and secretion from udder of an infected animal[6]. Approximately half of the total coliform count was attributed to *faecal coliforms* including *E.coli*. This indicates great possibility of the occurrence of enteric pathogens in milk[11].

E.coli and *Streptococci* are most common contaminants of faecal origin and could be an important factor of gastrointestinal infection including food poisoning and food borne illness[16]. Among all microorganism, *E.coli* is frequently contaminating organism and is reliable indicator of faecal pollution.

Microbiological indicators are commonly used in the assessment of public health risks associated with faecal contamination of freshwater ecosystems. Sediments are a reservoir of microorganisms, and can thus provide information on past pollution events, not obtainable through the testing of surface water. Moreover, pathogens present in sediment may represent future threats to human health. *Clostridium* spp, a typical colonizer of sediments, has been suggested as an alternative indicator of faecal pollution. In order to be suitable for such purpose, the microorganism may be widely distributed in contaminated environments.

Clostridia is an important pathogen in Veterinary and medical fields. Diseases caused by these organisms, are in many cases life threatening or fatal. C.*perfringens* type A food poisoning is one of the most common illness in the industrialized world. For the epidemiologist and veterinarian every *C.perfringens* occurrence is a rare and unforgiving challenge and a public health

emergency that requires rapid recognition and smooth co-operation between authorities to prevent additional cases.

Taken together, detection and inhibition of adulterants and pathogens in the milk is in need of the hour which will not only address the national need and export potential of Indian food processing industry. This comprehensive approach will add value by controlling the adulterated milk, after careful studies it can help in combating the microbial load.

MATERIAL AND METHODS

Sample collection: Cattle milk samples were from five different brands sold in Parbhani city; whereas buffalo milk samples were collected where from raw milk. All the five brands of cattle milk were of pasteurized milk. The brands of cattle milk were given code names to hide identity. The samples were collected aseptically as per standard procedure and protocols.

Application of phosphatase test:

Phosphatase test was done for pasteurized milk samples to check the efficacy of pasteurization as per the method described by Kay and Graham[14] with some modifications i.e. used a single dye for testing the samples.

Determination of total viable count (TVC):

Total viable count of milk samples was calculated as per the method described by Association of Official Analytical Chemists[2].

Determination of Differential count

Selective media were used for isolation of *E.coli*, *Streptococci*, *Clostridia* and *Salmonella*. Colony counts of bacterial isolates were taken as per the method described by American Public Health Association (APHA), 1984. Growth of *Clostridia* and *Salmonella* on selective media was recorded.

Identification and characterization of Isolates

The isolates obtained were further identified by using various staining reactions, biochemical tests and advance technique like Polymerase Chain reactions (PCR).

Biochemical characterization of isolates

The presumptive isolates were subjected to various biochemical tests as described by Cowan and Steel[4].

Sugar fermentation tests

Sugar fermentation tests were performed to detect production of acid and gas from Dextrose (D-glucose), Lactose, Maltose, Mannitol, Adonitol, Inositol, Raffinose, Salicin and Cellobiose in 1 per cent (w/v) Buffered Peptone Water mixed with phenol red indicator with inverted Durham tubes. Acid production was indicated by the color change from red to yellow and gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube[5].

Identification by Polymerase Chain Reaction

The Singleplex PCR of *E.coli* isolates was performed using various primers for the molecular characterization of isolates. The molecular characterization of isolates was done at National Veterinary Type Culture Center (VTCC), at National Research center on Equines (NRCE), Hissar (Haryana).

DNA extraction

DNA extraction of bacterial isolates was done as per standard method described by Sambrook *et al*.[22].

The polymerase Chain Reaction was for amplification of DNA extracted from *Pal* gene from isolated *E.coli* was done as per the method described by Peter *et al*.[21]. Cycle parameters were as follows.

(1) Initial denaturation at 95˚C for 5 minute
(2) Denaturation at 94˚C for 15 minute
(3) Annealing at 55˚C for1 minute
(4) Extension at 72˚C for 1 minute
(5) 34 cycles of steps 2 through 4 inclusive and
(6) Final extension at 72˚C for 10 minute

Table 1: Primers and cycling condition

Sr.No	Species	Gene	Primer	Size	Cycling condition
1	*E. coli*	*Pal*	F:5'-GGCAATTGCGGCATGTTCTTCC-3' R:5'-CCGCGTGACCTTCTACGGTGAC-3'	280bp	95ºC for 5 min followed by 35 cycles of 95°C1 min,60 ºC 30s, 72°C 30s and 72ºC for 10 min as final extension.

Electrophoresis

Two µl of PCR product was loaded in 1per cent agarose gel and run at 5v/cm a horizontal electrophoresis assembly (ATTO^{R} Japan). The expected PCR product of 791 bp was visualized using 0.5µg ethidium bromide in each ml of agarose under UV light in gel documentation system (Alpha IMAGER 3400 HP, USA). The gel was photographed and annotated using a PC based ALPHA IMAGER HP software.

Recording and handling of data

A data on microbial analysis of milk samples were analyzed by using Generalized Linear Model with the help of SYSTAT® software VERSION 7.0. Variations among all the group of all six sources were studied in relation to total viable count (TVC) and differential count (DC) of bacterial isolates.The isolates were submitted to National Veterinary Type Culture centre, National Research Center on Equines, Hissar (Haryana), for further typing and maintenance at Repository.

RESULTS AND DISCUSSIONS

A total of 198 milk samples collected from all six sources were processed for microbial analysis. The details are given in Table 1.

Table 1: Details of milk samples procured for microbial analysis

Sr. No.	Source	No of samples
1	Brand A	22
2	Brand B	22
3	Brand C	22
4	Brand D	22
5	Brand E	22
6	Raw milk	88
Total		**198**

Phosphatase test of Pasteurized milk samples

All the pasteurized milk samples of all the five brands were tested for pasteurization by using phosphatase test. All the samples tested were negative for phosphatase test indicating proper pasteurization of milk. The results are shown in Table 2.

Table 2: Results of phosphatase test of pasteurized milk samples

Sr. No.	Source	No. of samples tested	Result
1	Brand A	22	Negative
2	Brand B	22	Negative
3	Brand C	22	Negative
4	Brand D	22	Negative
5	Brand E	22	Negative
Total		**110**	

Total viable count of milk samples of different sources

Total viable counts (TVC) of all the milk samples were studied to assess the microbial quality of raw and pasteurized milk sold in Parbhani city. A total of 198 milk samples from all the sources were screened for TVC. Mean TVC were calculated. The results are shown in Table 3.

Table 3: Details of milk samples screened for microbial analysis (TVC)

Sr. No.	Source	No. of samples tested	Mean ± S.E. (cfu/ml)
1	Brand A	22	13.65 ± 0.19
2	Brand B	22	13.03 ± 0.21
3	Brand C	22	14.12 ± 0.19
4	Brand D	22	13.42 ± 0.20
5	Brand E	22	13.04 ± 0.19
6	Raw milk	88	14.99 ± 0.19
Total		198	

A completely Randomized Design is used for statistical analysis by using Generalized linear model by using SYSTAT® VERSION 7.0 program for comparison of mean TVC among all the sources. The results are presented in Table 4.

A significant (P<0.05) effect of different sources upon TVC was seen. Comparison among various sources revealed that raw milk is having significantly (P<0.05) high TVC than other sources. The mean TVC of brand C and raw milk are at par each other. A non significant difference in mean TVC counts was observed among brands A, B, C, D and E.

Table 4: Results of statistical analysis of Total Viable Count (TVC) of milk samples of different sources

Sr. No.	Source	Mean ± S.E. (Range)	F value	C.D.
1	Brand A	13.65^{b} ± 0.19 (11.79 - 15.51)	3.261*	1.171
2	Brand B	13.03^{b} ± 0.21 (11.54 - 14.52)		
3	Brand C	14.12^{ab} ± 0.19 (13.27 - 14.97)		
4	Brand D	13.42^{b} ± 0.20 (11.52 - 15.12)		
5	Brand E	13.04^{b} ± 0.19 (11.29 - 14.79)		
6	Raw Milk	14.99^{a} ± 0.19 (12.26 – 17.72)		

* - Significant - (P<0.05)

- Different superscripts show significant (P<0.05) difference between sources

It is interesting to note that though proper pasteurization of milk has been done (Table2). Presence of microbial load post pasteurization (Table 3) is an indicator of improper maintenance of temperature of milk during transportation and storage. Perusal of Table 4 indicate that comparison of mean TVC counts amongst sources reveal significant ($p<0.05$) differences amongst the sources. Thereby indicating contamination post production Tahira and Geetha[27] reported microbial load in pasteurized branded milk sold in Madurai city. Many earlier workers reported microbial load in raw milk[19,15,12,23,26]. Results of present study are on similar line.

Isolation of pathogenic bacteria from different sources

E.coli

E.coli counts of milk samples of all the sources were taken and mean counts were calculated. The results are presented in Table 5.

Table 5: Mean bacterial counts of *E. coli* of milk samples of different Sources

Sr. No.	Source	Mean ± S.E. (cfu/ml)
1	Brand A	$13.61^{b} \pm 0.18$
2	Brand B	$13.90^{b} \pm 0.18$
3	Brand C	$14.40^{ab} \pm 0.19$
4	Brand D	$13.62^{b} \pm 0.18$
5	Brand E	$13.54^{b} \pm 0.18$
6	Raw milk	$15.05^{a} \pm 0.19$

A Completely Randomized Design is used for statistical analysis by using Generalized linear model by using SYSTAT® VERSION 7.0 programme. The data was analyzed to find out the effect of different sources upon *E.coli* counts (Table 6). The source of milk was having significant ($P<0.05$) effect upon *E.coli* counts. Raw milk contain significantly high ($P<0.05$) *E.coli* counts than branded pasteurized milk samples. Critical observations of Table 6 revealed that brand C had significantly high ($P<0.05$) *E.coli* counts than other brands. It is interesting to note that the brands viz brand A, B, C, D and brand E were at par relation to *E.coli* counts.

Table 6: Results of statistical analysis of *E. coli* of milk samples of different sources

Sr. No.	Source	Mean ± S.E. (Range)	F ratio	C.D.
1	Brand A	$13.61^{b} \pm 0.19$ (11.69 - 15.55)	3.342*	0.927
2	Brand B	$13.90^{b} \pm 0.21$ (13.28 - 14.52)		
3	Brand C	$14.40^{ab} \pm 0.19$ (13.55 – 15.25)		
4	Brand D	$13.62^{b} \pm 0.20$ (12.77 - 14.47)		
5	Brand E	$13.54^{b} \pm 0.19$ (12.29 - 14.79)		
6	Raw Milk	$15.05^{a} \pm 0.19$ (12.32 - 17.78)		

* - Significant - ($P<0.05$)

- Different superscripts show significant ($P<0.05$) difference between sources

A total of 69 *E.coli* isolates were obtained from different sources. The overall percentage of samples positive for *E.coli* was 34.84 percent (Table 7).

Table 7: Percent Prevalence of *E.coli* of milk samples of different Sources

Sr. No.	Source	Total no of samples analyzed	Total No.of positive samples*	Percent positive samples
1	Brand A	22	02	09.09
2	Brand B	22	03	13.63
3	Brand C	22	08	36.36
4	Brand D	22	01	04.54
5	Brand E	22	02	09.09
6	Raw Milk	88	53	60.22
Total		**198**	**69**	**34.84**

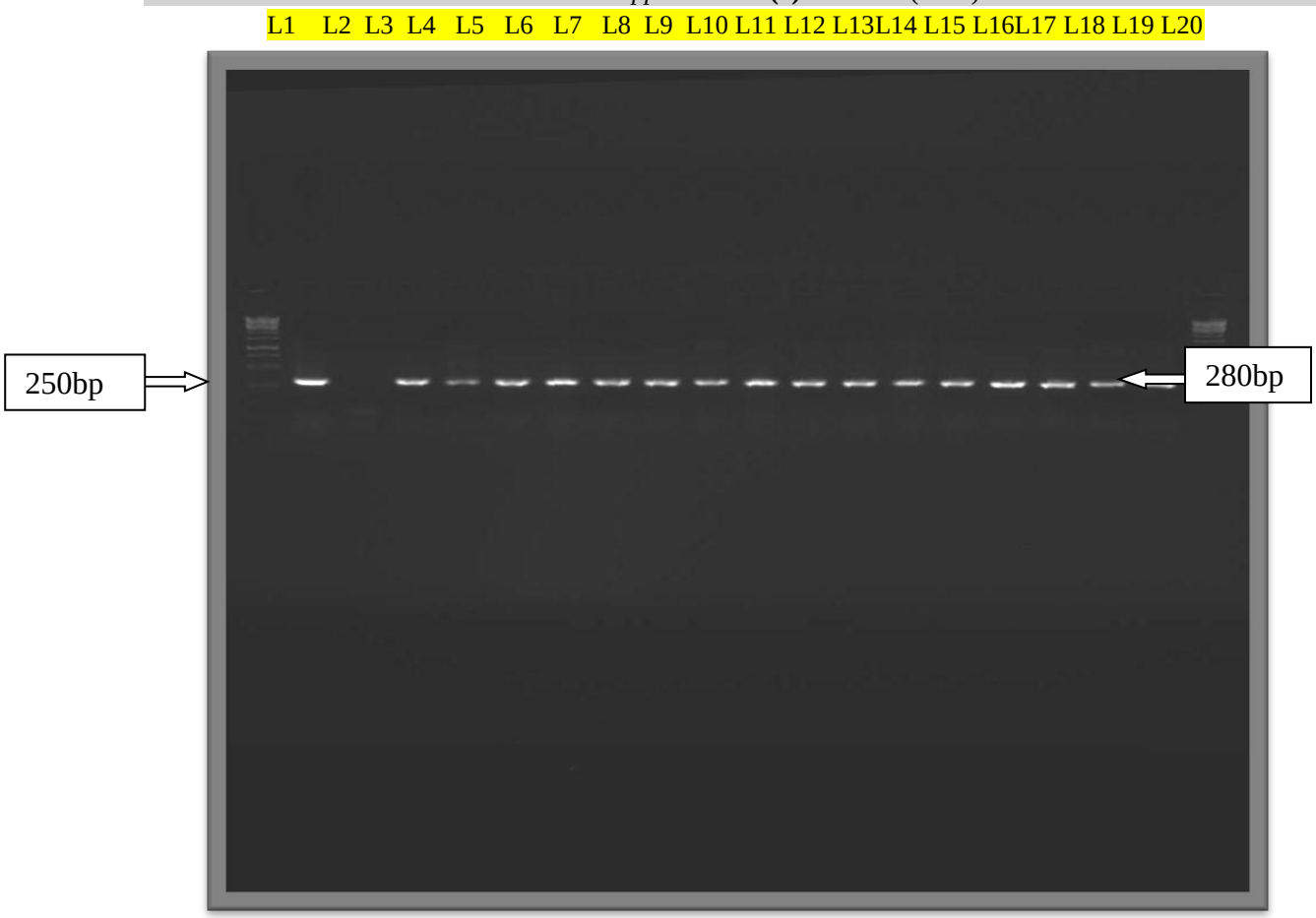
*- Positive for *E.coli* based upon staining reaction, biochemical and sugar fermentation tests

The confirmation of isolates was made by using biochemical and sugar fermentation tests. The highest (53) number of isolates were obtained from raw milk. Raw milk contain the highest (60.22) percentage of *E.coli* load. Earlier presence of *E.coli* in pasteurized and raw milk has been reported by many workers[25,11,20,16]. The results of present study are on similar lines.

Out of 69 *E.coli* isolates, a total of 16 isolates were further used for molecular characterization. The *pal* gene of *E.coli* was targeted for characterization. Singleplex PCR was used for extension of targeted genes of *E.coli* isolates. Electrophoretic patterns of marker gene and isolate gene were compared for confirmation. All the 16 *E.coli* isolates were found positive for the presence of *pal* gene, thereby confirming the *E.coli* isolates. The results are given in Table 8 and Plate 1.

Table 8: Results of molecular analysis of E.coli of milk samples of different sources

Sr. No.	Isolate code	Result of PCR*
1	A22	**Positive**
2	A24	**Positive**
3	B1	**Positive**
4	B15	**Positive**
5	B25	**Positive**
6	D10	**Positive**
7	D18	**Positive**
8	E7	**Positive**
9	E17	**Positive**
10	Rt 33	**Positive**
11	Rt 39	**Positive**
12	Rt 40	**Positive**
13	Rt 52	**Positive**
14	Rt 80	**Positive**
15	Rt 84	**Positive**
16	Rt 88	**Positive**



L1 and L20: 1kb DNA marker
L2: Positive control
L3: Negative control
L4-L20: PCR amplification of species specific *pal* gene of *E. coli.* Samples collected from milk samples

Plate1: Agar gel showing amplification of *PAL* gene isolated from *E.coli*

Earlier Sturgis successfully used *tol-pal* gene cluster for phylogenetic studies of *E.coli*. In present study also *pal* gene of *E.coli* has been successfully exploited for molecular characterization of *E.coli* isolates.

Streptococci

All the 198 milk samples were subjected to differential count for *streptococci* by using selective medium. Mean streptococcal counts of milk samples from different sources were calculated in Table 9.

Table 9: Mean bacterial counts of Streptococci of milk samples of different sources

Sr. No.	Source	Mean ± S.E. (cfu/ml)
1	Brand A	12.91 ± 0.20
2	Brand B	13.22 ± 0.20
3	Brand C	12.90 ± 0.20
4	Brand D	13.61 ± 0.22
5	Brand E	14.08 ± 0.20
6	Raw milk	15.55 ± 0.20

A completely Randomized Design is used for statistical analysis by using Generalized linear model by using SYSTAT® VERSION 7.0 programme to study the effect of various sources upon streptococcal counts. The results are given in Table 10. It is interesting to note that a non significant effect of sources of milk upon streptococcal counts was observed.

Table 10: Results of statistical analysis of Streptococci of milk samples of different sources

Sr. No.	Source	Mean ± S.E. (Range)	F ratio
1	Brand A	12.91^{a} ± 0.20 (11.92 - 13.89)	1.415^{NS}
2	Brand B	13.22^{a} ± 0.20 (11.92 - 14.52)	
3	Brand C	12.90^{a} ± 0.20 (12.05 - 13.75)	
4	Brand D	13.61^{a} ± 0.20 (12.20 - 15.02)	
5	Brand E	14.08^{a} ± 0.19 (11.93 – 16.22)	
6	Raw Milk	15.01^{a} ± 0.19 (13.98 – 16.04)	

NS – Not Significant

All the 198 samples were subjected to study the prevalence of streptococcal contamination by using staining reaction, biochemical and sugar fermentation tests. A total of 70 streptococcal isolates were obtained from different sources with overall positivity of 35.35 percent. The highest (50.00) percentage of streptococcal contamination was seen in Raw milk, whereas highest number of isolates (44) were obtained from raw milk. The results are shown in Table 11

Table 11: Percent prevalance of *Streptococci* in milk samples from different sources

Sr. No.	Source	Total no of samples analyzed	Total No.of positive samples*	Percent positive samples
1	Brand A	22	07	31.81
2	Brand B	22	04	18.18
3	Brand C	22	09	40.40
4	Brand D	22	02	09.09
5	Brand E	22	03	13.63
6	Retail Milk	88	44	50.00
Total		**198**	**70**	**35.35**

Positive for streptococci based upon staining reaction, biochemical and sugar fermentation tests.

Streptococcal contamination generally is of faecal origin indicating poor hygienic conditions. Earlier many workers reported streptococcal contamination. of milk[8]. The results of present study are in agreement with earlier work.

Clostridia

Clostridia are being used as an indicator organism for soil contamination of milk. Lingathurai and Vellathurai[18] studied clostridial contamination of cow milk. *Clostridia* could not be detected by them. Similarly Erickson *et al.*[8] also could not isolate *Clostridia* from milk samples.

It is interesting to note that a total of six clostridial isolates were obtained from different sources in the present study. The isolation was done by using SPS medium in anaerobic condition. Staining reaction, biochemical and sugar fermentation tests were used as criteria for confirmation of isolation. The overall prevalence of clostridia was low i.e. 3.03 percent. The highest (03) isolates were obtained in milk samples of brand C whereas brand E, B and raw milk did not have clostridial contamination.The results are shown in Table 12.

Table 12: Percent prevalence of *Clostridia* in milk samples from different sources

Sr. No.	Sample Code	Total no. Of samples analyzed	Total no of positive samples	Percent prevalance
1	Brand A	22	01	04.54
2	Brand B	22	00	00.00
3	Brand C	22	03	13.63
4	Brand D	22	02	09.09
5	Brand E	22	00	00.00
6	Raw milk	88	00	00.00
Total		**198**	**06**	**3.03**

*- Positive for clostridia bacteria upon staining reaction, biochemical and sugar fermentation tests.

Salmonella

Ekici *et al.*[7] attempted *Salmonella* isolation from 66 raw milk samples, however *Salmonellae* could not be isolated. Similarly, Chandra Shekhar *et al.*[3] also could not isolate *salmonellae* from milk in Uttar Pradesh. Ghose and Maharajan[10] also attempted to isolate *salmonellae* from raw milk in Bangladesh but failed to isolate the *salmonella*. However, Hossain *et.al,*[13] showed presence of *salmonella* in raw milk. Similarly Lingathurai and Vellathurai[18] detected *Salmonella* in 8 samples out of total 60 samples analyzed with the 13.3 percent prevalence from raw milks of Madurai city.

In present study, screening of all 198 milk samples from different sources revealed absence of *salmonella*. The results are on similar lines reported earlier.

Quality

In the present study, despite of negative phosphatase test of all the branded processed milk sold in Parbhani city, TVC counts were observed among them. Comparative microbial analysis of branded and raw milk indicates that raw milk contain higher bacterial load than branded one .Pathogenic bacteria like *E.coli, Streptococci, Salmonella and Clostridia* are being taken as indicator organisms for microbial quality assessment. *E.coli* and *Streptococci* contamination was rampant amongst processed branded as well as raw milk .In recent times PCR technique is being widely used for characterization of *E.coli.*

In present study Singleplex PCR has been successfully exploited for characterization of isolates. In recent times, microbes possess importance in relation to proprietary. A National Veterinary Type Culture Center (NVTCC) has been started by Govt. of India at National Research Center on Equines (NRCE), Hissar (Haryana). All the isolates of E.coli isolated in present study were submitted for further typing and maintenance at NVTCC (NRCE) as Repository.

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