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Studies on detection and antibiotic sensitivity and resistance pattern of *Listeria monocytogenes* isolated from cattle, raw cow milk and milk products from Tripura, India

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Abstract
A total 200 numbers of samples including cattle faeces (50), raw milk (50) and milk products (100) were collected randomly to study the occurrence and antibiotic sensitivity of *L. monocytogenes* from West Tripura district, Tripura. The *L. monocytogenes* was isolated by using two step enrichment method of culturing and identified based on cultural characteristics, gram staining, biochemical properties, tumbling motility and *in vitro* pathogenicity tests. The antibiotic sensitivity was studied against 12 numbers of commonly used antibiotics in animals and human. The occurrence of *L. monocytogenes* was recorded as 8.50 percent including 6.00 percent (3/50) from cattle faeces, 8.00 percent (4/50) from raw milk, 12.00 percent (3/25) from lassi, 16.00 percent (4/25) from dahi and 12.00 percent (3/25) in ice-cream. The *L. monocytogenes* strains showed 100 percent sensitivity towards Penicillin, Ampicillin, Oxacillin, Cephotaxime /Clavulanic acid, Ciprofloxacin, Tetracycline and Trimethoprim/Sulphamethoxazole followed by Streptomycin (88.23%), Chloramphenicol (82.35%), Gentamicin (70.58%) and Ceftriaxone (52.94%). The presence of the organism in milk and milk products indicates unhygienic handling during production, processing and marketing is an alarming public health threat to the consumers.

Keywords: *Listeria monocytogenes*, occurrence, antibiotic sensitivity, West Tripura

1. Introduction
As a consequence of rapid globalization, the security and safety of food gradually have become the major issue of health concern in developed as well as developing countries due to changing food habits all over the world. India is one of the most advanced milk producers in the world. Fifty percent of the total produced milk is being consumed by rural households and the rest is sold in the domestic market, out of which, 50 percent as fluid milk, 35 percent as traditional products and 15 percent as other processed dairy products (Himabindu *et al*., 2014) [14]. Majority of the population of India have diversified food habits and consumption of dairy products is primarily skewed towards traditional ones. The milk and dairy products are perishable and they are susceptible to microbial contamination especially under tropical and unhygienic conditions of production, storage and selling prevailing in the unorganized sector of milk industry in India.

Listeriosis is one of the most important bacterial zoonotic infections mainly caused by *Listeria monocytogenes* and it is categorized under List C of OIE disease. During last 25 years, *L. monocytogenes* has become increasingly important as a food-associated pathogen and has been listed among the most frequent causes of death due to food-borne illness in human beings CDC (2003) [6]. *Listeria monocytogenes* has been identified as a causative agent in series of outbreaks of human listeriosis with high mortality rate (30%) involving milk and dairy products (Boujemaa *et al.*, 2013) [5].

Listeria monocytogenes is ubiquitous, Gram-positive, rod-shaped, non spore-forming and facultative anaerobic bacteria with the special growth characteristics of growing in pH range of 4.4 – 9.4, resisting high salt levels, nitrite and acid. The organism is relatively resistant to drying but can easily be destroyed by heating. It can grow between 0°C – 45°C (Sue *et al*., 2009) [26].

Healthy cattle are capable of serving as reservoirs for *L. monocytogenes* by excreting the organism through their milk.

Milk can also become contaminated through accidental contact with faeces and silage. It is also documented that products prepared from raw milk are having *L. monocytogenes*. The consumption of raw milk or products made of raw milk has caused several listeriosis outbreaks resulting in hundreds of cases (Pal and Awel, 2014) [24].

The studies on *L. monocytogenes* in the perspective of foodborne pathogen are scanty in North-East Region states including Tripura. Therefore keeping the above points in view, the present study was undertaken to isolate, identify and to study the prevalence and antimicrobial sensitivity pattern of *L. monocytogenes* from different samples of cattle source s of West Tripura district (Tripura).

2. Materials and Methods

2.1 Study area

The present study on isolation and identification, occurrence and antimicrobial sensitivity pattern of *L. monocytogenes* from different samples of cattle sources was carried out in West Tripura district of Tripura. Tripura covers 10,491 km² of land area and is bordered by Bangladesh to the north, south and west and the states of Assam and Mizoram to the east. Tripura is situated between the latitudes of 22°56´N to 24°32´N and the longitudes of 90°09´E to 92°10´E. The state has 60 percent hilly land and 40 percent plains with heavy rainfall (an average rainfall of 210 cm per annum) and sub-tropical climate. Livestock is an important component of agricultural system that plays a vital role in determining the agricultural economy by providing gainful employment, particularly to the small and marginal farmers, women and agricultural labourers in Tripura. Tripura stands second in total livestock population (1869 thousand) occupying second position in cattle (954 thousand) in the North-East Region (Livestock Census. 2003) [15].

2.3 Period of the study

The study was conducted for a period of one year from July, 2017 to June, 2018 in West Tripura district of Tripura, India and the study period was divided into two halves; Summer (March to September) and Winter (October to February).

2.4 Collection of samples

A total 200 number of samples including cattle faeces, raw milk and milk products were collected randomly for detection of *L. monocytogenes* from different unorganized farms/ milk vendors/ shop by following aseptic measures at periodic intervals during the study period. Distribution of different samples collected is given in the Table-1 and Figure-1 and 2.

Table 1: Distribution of different samples of cattle sources collected from West Tripura district of Tripura

Sl. No.	State	Sample	Number of sample	Seasonal distribution	
				Summer	Winter
1	West Tripura (Tripura)	Cattle faeces	50	25	25
2		Raw cow milk	50	25	25
3		Lassi	25	13	12
4		Dahi	25	12	13
5		Ice-cream	25	13	12
6		Rasmalai	25	12	13
Total			200	100	100

Fig 1: Unhygienic milking practices

Fig 2: Unhygienic milk storing practices

2.5.1 Enrichment of faecal sample

The USDA (USDA FSIS, 2002) [30] method was employed for isolation of *Listeria spp*. from faecal samples of cattle by two step enrichment method. Primary enrichment of five grams of faecal sample was done in 45 ml 1/2 strength UVM-I broth containing selective supplements (HiMedia Pvt. Ltd., Mumbai) and incubated for 24 hours at 30°C followed by secondary enrichment of 0.1 ml from the broth culture in 10 ml UVM-II broth containing selective supplements (HiMedia Pvt. Ltd., Mumbai) and incubated for 48 hours at 37°C.

2.5.2 Enrichment of milk and milk products

For isolation and identification of *L. monocytogenes* from cattle faeces, raw milk and milk products (lassi, dahi, ice-cream and rasmalai), Food and Drug Administration (2015) [12] testing methodology with slight modification was employed. Twenty five ml of sample was mixed with 225 ml of UVM broth properly for two minutes and the mixture was incubated at 30°C for 24 hours. For secondary enrichment, 0.1ml of the cultured UVM was transferred to 10 ml of Fraser broth (FB) and incubated at 37°C for 24hours.

2.5.3 Selective plating of UVM-Broth and FB culture in PALCAM, McBride and TSYEA agar

A drop of approximately 0.1 ml of FB broth culture turning to black colour was streaked aseptically upon a PALCAM and McBride agar plate and the plate was incubated at 37°C for 24 - 48 hours. The suspected colonies on PALCAM/ Mc Bride agar plates were streaked on TSYEA plate with the help of a sterile loop and incubated at 37°C for 24 hours and subsequently tested for further biochemical and *in vitro* pathogenicity characteristics.

2.5.4 Morphological and biochemical characteristics of *L. monocytogenes*

The *L monocytogenes* were phenotypically characterized based on morphological characteristics, Gram staining reaction and biochemical characteristics (Catalase, Oxidase, Motility, Indole, Methyl Red, Voges-Proskauer, Citrate utilization, fermentation patterns of sugars like L-Rhamnose, D-Xylose and Mannitol) (Quinn *et al.*, 1994) [25] (Table-2).

Table 2: Details of different biochemical characteristics of *L. monocytogenes*

Test	Indicator reagent	Colour before reaction	Colour after reaction	Observation (positive cases)
Catalase	3% H_2O_2	Colourless	White effervescence	Positive
Oxidase	-	White	Purple	Negative
Motility	-	Cream	Movement of the growth with simultaneous blackening	Tumbling
Indole	Kovac's Reagent	Light Yellow	Pink Red ring	Negative
Methyl Red	1-2 drops of methyl red	Colourless	Red	Positive
Voges-Proskauer	1-2 drops of Baritt reagent A and Baritt reagent B	Light yellow/colourless	Pinkish red	Positive
Citrate	-	Green colour	Blue colour	Negative
L-Rhamnose fermentation	-	Pinkish red/Red	Yellow	Positive
D- Mannitol fermentation	-	Pinkish red/Red	Yellow	Negative
D- Xylose fermentation	-	Pinkish red/Red	Yellow	Negative

2.6 *In vitro* pathogenicity test

2.6.1 Beta haemolysis test on five percent sheep blood agar (SBA)

The suspected colonies of *L. monocytogenes* on PALCAM/ McBride agar plate and TSYEA agar plate were touched with a sterile loop and streaked on five percent SBA plates and the plates were incubated at 37°C for 24 hours. The *L. monocytogenes* on SBA plate appeared as translucent colonies surrounded by a small zone of β-haemolysis after back light.

2.6.2 Christie, Atkins, Munch- Petersen (CAMP) test

The presence of *in-vitro* pathogenicity of *L. monocytogenes* by CAMP test was tested as per the method of ISO (1996) [16]. The standard strains of *Rhodococcus equi* (MTCC 8144) and *Staphylococcus aureus* (MTCC 43300) were streaked on freshly prepared five percent SBA plates wide apart and parallel to each other. The test strains were streaked at 90° angle to *R. equi* and *S. aureus* with a distance of three mm apart from these strains streaking line. The streaked plates were incubated for 24 hours at 37°C and examined for haemolytic zone from partial haemolysis to a wider zone of complete haemolysis. The isolates with CAMP positivity against *S. aureus* were characterized as *L. monocytogenes* giving a spade shaped haemolytic zone formation.

2.7 Detection of antibiotic sensitivity and resistance pattern of *L. monocytogenes* strains

All the *L. monocytogenes* isolates were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method (Bauer *et al*.,1966) [3] against a panel of 12 antibiotics namely Penicillin G, Ampicillin, Oxacillin, Streptomycin, Erythromycin, Cephotaxime/Clavulanic acid, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Gentamicin, Tetracycline and Trimethoprim/Sulphamethoxazole as per Clinical and Laboratory Standard Institute (CLSI) guidelines [8]. The *L. monocytogenes* isolates were inoculated into Brain Heart Infusion (BHI) broth and incubated for 24 hours at 37°C and 200 µl of each broth inoculum was taken on Muller Hinton Agar plates and spread eventually with the help of sterile L-shaped spreader. Then the plates were allowed to dry and antibiotic discs were placed on media aseptically with the help of sterile forceps. The plates were incubated at 37°C for 24-48 hours and the diameter of zone of inhibition was compared with the standard known value against each specific antimicrobial agent from interpretation guide line (Hi-Media).

3. Results and Discussion

3.1 Isolation and identification of *L. monocytogenes*

Out of 200 different samples from cattle sources (cattle faeces, raw milk and milk products) of West Tripura district of Tripura, a total 36 (18.00%) samples were found to be positive for *Listeria spp.* by the cultural method in which isolates turned into black colour in different broth (FB and UVM) and also showed characteristics of colonies on different agars such as green colonies with black holoes in PALCAM agar, dense white to iridescent white appearing as crushed glass in McBride agar and clean glass like colonies in TSYEA agar after 24-48 hours of incubation at 37°C. Based on the positive Gram staining reaction, tumbling motility, different biochemical tests (catalase: positive:; oxidase: negative: tumbling motility; indole: negative; methyl red: positive; Voges-Proskauer: positive citrate: negative; L-rhamnose: positive; D- Mannitol fermentation: negative; D-Xylose fermentation: negative), weak haemolysis on SBA and positive CAMP test against *Staphylococcus aureus*, 17(8.50%) numbers of *L. monocytogenes* were identified and the findings were in accordance with Walse *et al.* (2003) [31] and Gupta and Sharma (2012) [13](Table-3 and Figure- 3, 4, 5 and 6).

Table 3: Detection of *L. monocytogenes* by cultural method from different samples of cattle sources from west Tripura district of Tripura

Sl. No.	State	Type of sample	Number of sample analyzed	Number of sample positive for *Listeria spp.* by cultural method	Number of sample positive for *L. monocytogenes* by phenotypic characteristics
7	West Tripura (Tripura)	Cattle faeces	50	9	3(6.00)
8		Raw cow milk	50	8	4 (8.00)
9		Lassi	25	5	3 (12.00)
10		Dahi	25	7	4 (16.00)
11		Ice-cream	25	6	3 (12.00)
12		Rasmalai	25	1	0 (0.00)
Total			200	36 (18.00%)	17 (8.50%)

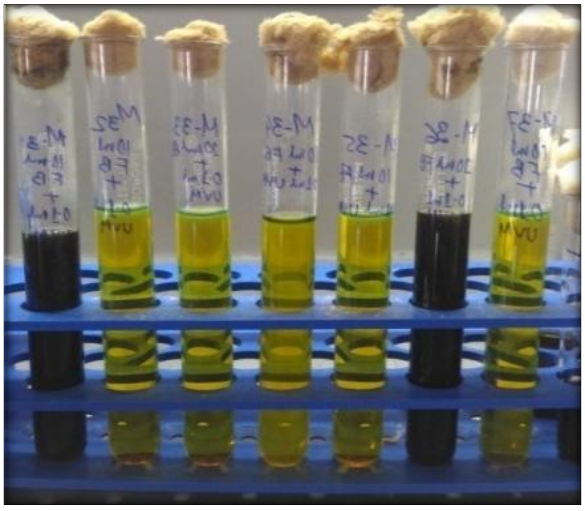

Fig 3: 10 ml FB broth containing 0.1 ml cultured UVM broth

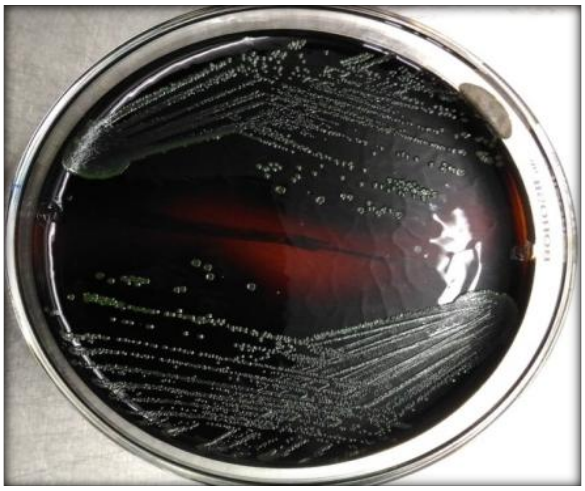

Fig 4: *L. monocytogenes* on PALCAM agar

Fig 5: *L. monocytogenes* on McBride agar

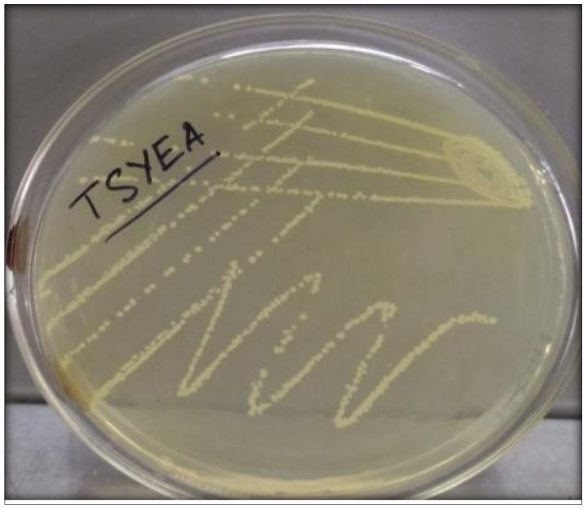

Fig 6: *L. monocytogenes* on TSYEA agar

The detection of *Listeria* spp. from food products is challenging due to the concurrent presence of other organisms within the food product. In this respect, the isolation method in respect to specific pathogen is critical and must allow recovery and detection of injured cells too. In food, detection of *Listeria* spp. is generally performed in a two-step cultural enrichment process and along with selective supplements like antibacterial and antifungal agents. The bacteriological culture methods commonly used for detection and identification of the bacteria include aesculin and ferric iron in enrichment or plating media which results through the hydrolysing capacity of *Listeria* spp. with the formation of intense black colour (Fraser and Sperber 1988) [10]. Results of *in vitro* pathogenicity tests showed that *Listeria* spp. brought about haemolysis on five percent SBA similar to the earlier records of Blanco *et al*. (2008) [4]. The Christie Atkins Munch-Petersen (CAMP) test is a unique confirmatory tool for identification of this food borne pathogen. The *Listeria* spp. isolates recovered during the study have shown the positive CAMP pattern against *S. aureus* (ISO 1996) [16].

3.2 Occurrence of *L. monocytogenes* in different samples of cattle sources (faeces, raw milk and milk products) from West Tripura (Tripura) district

The occurrence of *L. monocytogenes* was recorded as 8.50 percent (17/200) *comprised* of 6.00 percent (3/50) in cattle faeces, 8.00 percent (4/50) in raw milk, 12.00 percent (3/25) in lassi, 16.00 percent (4/25) in dahi and 12.00 percent (3/25) in ice-cream. *Listeria monocytogenes* could not be isolated from the milk product, rasmalai. *Listeria monocytogenes* was detected from the raw milk and ready to eat refrigerated milk products produced locally from unpasteurized milk like dahi, lassi and ice cream where as the organism was not isolated from rasmalai which is a well cooked milk product stored for a short duration of time in the sweet shops. The higher

occurrence rate of *L. monocytogenes* from faecal samples of ruminants were recorded by Lawan *et al.* (2003) [20] (10.00%) and Kalorey *et al.* (2006) [19] (16.00%) from Nigria and Nagpur, India, respectively. Waghmare (2006) [32] evaluated the incidence of *Listeria spp*. in raw milk from different markets of Mumbai city (India) and revealed prevalence of *Listeria spp*. and *L. monocytogenes* amongst the pasteurized milk samples with the incidence of 21.32 and 5.88 per cent in unpasteurized milk samples. Similarly Chandio *et al.* (2007) [7] reported 6.00 percent of *L. monocytogenes* in raw cow milk where as higher incidence of prevalence of *L. monocytogenes* (21.70%) was reported by Sharma *et al*. (2012) [28] from 115 raw cow milk samples in Meerut and Babugarh Cantt, Hapur, India. In contrast, studies conducted at Coimbatore (Tamilnadu) and Mangalore, India reported that branded milks were more prone to *L. monocytogenes* than the local milk (Dhanashree *et al.*, 2003 [9] and Sheela and Muthukmar 2011) [27]. Moharram *et al.* (2007) [21] reported 5.00 percent incidence of *L. monocytogenes* from non branded ice-cream samples from different ice cream parlours of Mysore (India).

The seasonal distribution of *L. monocytogenes* revealed 3.50 and 5.00 percent of prevalence in summer and winter season, respectively.The Seasonal fluctuation of *L. monocytogenes* in the milk has also been reported as 1.69 percent in summer and 3.82 percent in winter by Aurora *et al*. (2006) [1].

Listeria is a widely distributed bacterium in nature and commonly found in soil, sewage, dust, water and causes listeriosis in humans and animals (Norton *et al*., 2001) [22]. Of the various milk pathogens, *L. monocytogenes* is one of the deadly organisms that occurs largely in all types of environment including foods grown in contaminated environment, poorly processed/stored food, milk and associated products (Priyanka and Alka 2008) [23]. The study of incidence of *Listeria* spp. in cattle faeces, milk and milk products in their selling units provide information about the carrier status in cattle and contamination status of the milk and milk products. The milk producing and processing environment and handling practices may vary place to place and production practices. There are chances of increase in cross contamination as 47 percent of surface of hand of the food handlers and 16 percent on the processing tables were found to carry *L. monocytogenes* (Kerr *et al*., 1993 [18]; Jayasekaran *et al*., 1996) [17]. The presence of *Listeria* spp. particularly *L. monocytogenes* in ready to eat milk products like dahi, lassi, ice cream and raw milk could be a potential risk for consumers. As per requirements of the US-FDA, *L. monocytogenes* should be absent in RTE foods (Fusch and Reilly1992) [11].

3.3 Antibiotic sensitivity pattern of *L. monocytogenes*

All the 17 *L. monocytogenes* strains showed 100 percent sensitivity towards Penicillin, Ampicillin, Oxacillin, Cephotaxime /Clavulanic acid, Ciprofloxacin, Tetracycline and Trimethoprim/Sulphamethoxazole followed by Streptomycin (88.23%), Chloramphenicol (82.35%), Gentamicin (70.58%) and Ceftriaxone (52.94%). Conversely the *L. monocytogenes* strains showed highest resistance to Erythromycin (88.23%), Ceftriaxone (47.05%), Gentamicin (29.41%) and Chloramphenicol (17.64%), respectively (Table-4 and Figure-7 and 8).

Table 4: Antibiotic sensitivity and resistance pattern of *L. monocytogenes* isolated from different samples of cattle sources from West Tripura (Tripura) district

Sl. No	Antimicrobial agent	No. of isolates	Sensitive (%)		Intermediate (%)		Resistance (%)	
1	Penicillin G (P)	17	17	100	-	-	-	-
2	Ampicillin (AMP)	17	17	100	-	-	-	-
3	Oxacillin (OX)	17	17	100	-	-	-	-
4	Streptomycin (HLS)	17	15	88.23	2	11.76	-	-
5	Erythromycin (E)	17	-	-	2	11.76	15	88.23
6	Cephotaxime / Clavulanic acid (CEC)	17	17	100	-	-	-	-
7	Ceftriaxone (CTR)	17	9	52.94	-	-	8	47.05
8	Chloramphenicol (C)	17	14	82.35	-	-	3	17.64
9	Ciprofloxacin (CIP)	17	17	100	-	-	-	-
10	Gentamicin (GEN)	17	12	70.58	-	-	5	29.41
11	Tetracycline (TE)	17	17	100	-	-	-	-
12	Trimethoprim/Sulphamethoxazole (COT)	17	17	100	-	-	-	-

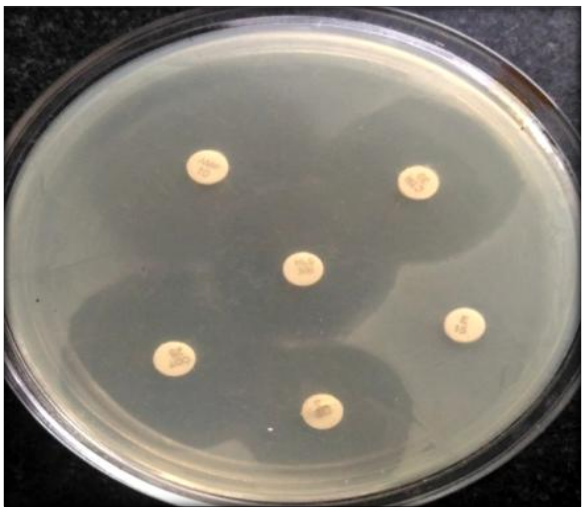

Fig 7: Antibiotic sensitivity and resistance pattern of *L. monocytogenes*

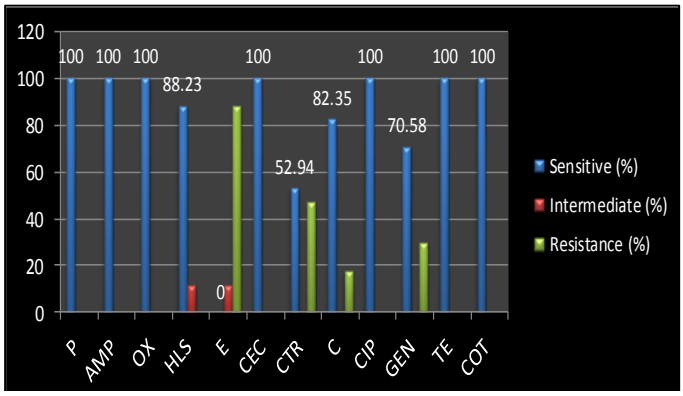

Fig 8: Antibiotic resistance and sensitivity pattern of *L. monocytogenes* strains obtained from West Tripura district

There is growing concern of bacterial adaptation and evolution resulting in the emergence of resistant bacterial

pathogens since last 50 years. The prevalence of antimicrobial resistance among food borne pathogens has increased during recent decades (Akbar and Anal 2014) [2]. The frequent and unnecessary use of antimicrobial agents in food animals for therapeutic and prophylactic purposes in animals are contributing to create resistant strains. Animal origin foods are the major sources of transmission of antimicrobial resistant organisms to human. The antimicrobial resistant bacteria from food animals may colonize the human population via food chain, contact through occupational exposure or waste run off from animal production facilities. Resistant bacteria may readily transferred from food animals to human beings as the similar kind of antimicrobial agents are used in human practice also, therefore the detection of antimicrobial resistance pattern is a matter of public health significance. Sharma *et al.* (2012) [28]detected80-90 percent resistance of *L. monocytogenes* strains from raw milk of Meerut and Babugarh Cantt, Hapur (India) to Nalidixic acid, Amoxycillin+Sulbactum, Vancomycin, Kanamycin, Cloxacillin, and Erythromicin whereas many were susceptible to the Ampicillin, Ofloxacin, Tetracycline, Streptomycin, Sulphafurazole, Oxacilin and Ciprofloxacin. The findings of Sharma *et al.*, (2017) [29] is alarming as they recently isolated Multi Drug Resistant (MDR) strains of *L. monocytogenes* from raw milk in Rajasthan and emphasized on the need of awareness among consumers. Implementation of food safety regulations at different levels of milk production has come up as a great public health issue.

4. Conclusion

The present study indicated the prevalence of *L. monocytogenes*, a major zoonotic pathogen causing fatal infections in human, in different samples of cattle sources namely faeces, raw milk and milk products in West Tripura district of Tripura indicated the public health significance of this pathogen. The presence of the organism in cattle faeces indicated the carrier status, presence in raw milk and refrigerated milk products produced locally and sold in local markets under unhygienic condition is an alarming public health threat to the consumers. The well cooked milk product (Rasmalai) which is stored for a short period of time has been found to be free from *L. monocytogenes.*

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