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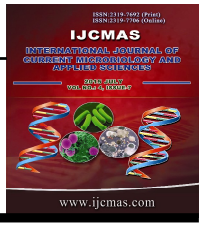

Original Research Article

Studies on the Prevalence of *Listeria Monocytogenes* in Unpasteurized Raw Milk Intended for Human Consumption in and Around Kolkata, India

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K e y w o r d s

Listeria monocytogenes, *Listeria* spp, Milk prevalence, Antibiotic sensitivity

A B S T R A C T

Listeria monocytogenes is a facultative anaerobic gram positive intracellular pathogen which infects cattle and is often secreted in cow's milk possessing serious health hazards to human consuming the milk. It causes up to 24% mortality in human (Farber and Peterkin, 1991). This study was undertaken to determine the prevalence of *Listeria spp*. in unpasteurised raw milk procured from, in and around Kolkata using two step enrichment procedures. For this, a total of 104 milk samples [from individual cow's udder (n=36) and from pooled can milk, collected from farm (n=20) as well as from market (n=48)] were examined for a period of 6 months starting from January, 2014 to June, 2014. Of the total milk samples examined 14 (13.46%) were found positive on culture on selective media as well as on gram staining for *Listeria spp*. On the basis of biochemical tests and other tests like CAMP test and Beta heamolysis, 5 (4.81%) isolates of *L. monocytogenes* were confirmed out of 14 suspected *Listeria spp*. The study concluded that overall prevalence of *Listeria spp*. and *L. monocytogenes* was found to be 13.46% and 4.81%. All the five *L. monocytogenes* isolates were subjected to antibiotic sensitivity test using Kirby-Bauer disc diffusion method. In this method 3 antibiotics, tetracycline, gentamicin, penicillin, exhibited complete sensitivity.

Introduction

L. monocytogenes is the most important species in the genus *Listeria* creating human health hazard and having a worldwide distribution with an extensive host range which includes mammal, poultry, fish, crustacean and ticks. *L. monocytogenes* is known to be secreted in milk by both infected and healthy animals (Wagner *et al.*, 2000). Human listeriosis is a food-borne disease, and it has been estimated that 99% of all human listeriosis cases are caused by consumption of contaminated food products (Mead *et al.*, 1999). Although listeriosis is not common in humans, it is a clinically significant disease because of its high mortality and severity (Atil *et al*., 2011).

L. monocytogenes has been called an "emerging food-borne pathogen" because only recently we have recognized that it can be transmitted through food. *L. monocytogenes* is a ubiquitous bacterium. It

causes listeriosis, a serious infectious disease which occurs as a consequence of consumption of food contaminated with this pathogenic bacterium. Listeriosis is a significant public health problem (Rocourt and Catimel, 1985). *L. monocytogenes* is associated with septicaemia, meningo-encephalitis and abortion in humans and animals, primarily affecting pregnant, new-born, and immune-compromised individuals (Choi and Hong, 2003; Rossmanith *et al.*, 2006). Several outbreaks of listeriosis were proven to be associated with the consumption of milk and causing great concern in the dairy industry due to the number of cases and the nearly 30% overall mortality rate of these outbreaks (Amagliani *et al*., 2004).

Materials and Methods

Samples

Raw milk samples were collected from the dairy farm directly from the udder as well as from the milk collection cans from different parts of Kolkata, West Bengal and its periphery for a period of 6 month starting from January, 2014 to June, 2014. Pooled bulk tank milk was also collected from different local markets.

Isolation of *Listeria Spp.* from raw milk

Isolation of *Listeria spp.* from the raw milk samples was attempted as per the US Department of Agriculture (USDA) method described by McClain and Lee (1988) after some necessary modifications. The method is briefly described below.

About 25 ml of milk sample was directly inoculated into 225 ml of University of Vermont Medium I (UVM I) and incubated overnight at 30°C. The enriched UVM I inoculum (0.1 ml) was transferred to UVM II medium then incubated overnight at 30^0_C.Then the enriched inoculum from UVM II was streaked on polymixin acriflavin lithium chloride ceftazidime aesculin mannitol (PALCAM) agar (Himedia® Mumbai, India). The inoculated plates were incubated at 37°C for 24–48 hrs. Grey green colonies with black sunken centers from PALCAM were suspected to be of *Listeria spp.* Then the presumed colonies of *Listeria spp.* (at least 3/plate) were sub cultured for further confirmation. Preliminary identification was done by observing colony characteristics, Gram staining and confirmatory identification was done by biochemical test.

Another method was followed to confirm the *Listeria spp*. isolates. The method is described briefly, all the milk samples were subjected to, isolation of *Listeria spp.* as per method described by EN-ISO 11290-1(ISO, 1996). The samples were subjected to primary enrichment by using 10 ml of milk samples with 90 ml Fraser broth. Then incubated at 30°C for 24 hrs. Then the culture was subjected to Secondary enrichment by adding 10 ml Fraser broth with 0.1 ml Primary enrichment Sample, then incubated at 37°C for 48 hrs. Plating was done by streaking of enriched culture on PALCAM *Listeria* selective media and incubated at 37°C for 24–48 hrs. *Listeria* colonies will be seen as gray-green colonies with black halo. Five presumptive colonies were selected for confirmation, where well separated colonies are not available. Streaking of one colony on Tryptone soya yeast extract agar was performed. *Listeria* colonies were confirmed by gram staining, Catalase test, Oxidase test, motility test, carbohydrate fermentation test, Beta haemolysis and CAMP test.

Microscopic identification was done by placing a loop full of colony from PALCAM agar on a grease free slide

containing one drop of sterile distilled water and a smear was prepared. The smear was subjected to gram staining and the slide was viewed using a compound microscope under oil immersion. Positive *Listeria spp.* cultures were identified as purple colour bacilli in single and chain form.

For biochemical Characterization following tests were done. Catalase test: A small amount of culture is picked up from the PALCAM agar by a sterile platinum loop and inserted into 3% H2O2 solution held in a small clean tube. In positive case, there is rapid evolution of oxygen (within 5–10 sec.) as evidenced by bubbling. A negative result is no bubbles or only a few scattered bubbles. Oxidase test was carried out by touching and spreading a well isolated colony on the oxidase disc (Himedia®). In positive cases, there is formation of deep purple colour within 5–10 sec. A change after than 10 seconds or no change at all is considered negative reaction.

Sugar fermentation test was carried out by taking 5 ml peptone water with andrade indicator (0.5%) was taken in each test tube containing Durham's tube. Then tube were sterilised under autoclave at 121°C for 15 minutes. On cooling to 45–50°C, a single carbohydrate disc namely Xylose, Rhamnose and Mannitol (Himedia®) was added to each tube aseptically and inoculated with the test organisms. Incubation was carried out at 37°C for 18–48 hrs. Results were recorded at 18–24 hrs and again at 48 hrs. Methyl red test was carried out by dispensing by 2 ml MR-VP Medium (Glucose Phosphate Broth) in each test tube and sterilised by autoclaving at 121°C. Small amount of culture from a young agar slope was inoculated into each test tube containing the medium and incubated at 30°C for five days. Then about 3–4 drops of methyl red indicator was mixed to each test tube and effect was read immediately. Positive test showed bright red and negative were yellow. Voges-Proskauer test, 2 ml MR-VP Medium (Glucose Phosphate Broth) was dispensed in each test tube and sterilised by autoclaving. Small amount of culture from a young agar slope was inoculated into each test tube containing the medium and incubated at 30°C for 24–48 hrs. 1 ml of Barritt Reagent A (R029 - 5% a-naphthol in absolute ethanol) 0.4 ml of Barritt Reagent B (R030 - 40% potassium hydroxide) added to the culture containing medium. Positive test is indicated by eosin pink colour within 2–5 minutes. CAMP test was done by incubating a standard strains of *Staphylococcus aureus* and *Rhodococcus equi* were grown overnight on 5% sheep blood agar (SBA) plates at 37°C and their colonies were again streaked on freshly prepared SBA plates having 5% sheep blood in a manner that these were wide apart and parallel to each other.

In between the parallel streaks of *S. aureus* and *R. equi*, *L. monocytogenes* isolates were streaked at 90 degree angle and 3 mm apart before incubating them at 37°C for 24 hrs. The plates were examined for enhancement of haemolytic zone from partial haemolysis to wider zone of complete haemolysis, any, between a *Listeria* strain and the *S. aureus* or *R. equi* strain owing to the synergistic effect of their haemolysins in case of a CAMP-positive reaction. *Listeria spp.* isolates showing CAMP-positivity only with *S. aureus* but not with *R. equi* were characterized *as L. monocytogenes.* Beta haemolysis, all the *Listeria* isolates that were confirmed using biochemical tests were analyzed for the type of haemolysis on Sheep blood agar (SBA) as per the method described by Seeliger and Jones (1986). The isolates were streaked onto 5% SBA plates and incubated at 37°C for 24 hrs and examined for haemolytic zones around the colonies. The characteristic β-

haemolysis in the form of wider and clear zone of haemolysis represented *L. ivanovii* while, a narrow zone of β-haemolysis was the characteristic of *L. monocytogenes*.

Scanning electron microscopy

Scanning Electron Microscopy was performed as per method described by Dewar (1982) with slight modification, which was done in the Central Instrument Department at Bardowan University, is described briefly; cultured broth was centrifuged at 12000X g for 10 minutes. Then the cell pellet was washed twice with PBS and the cell pellet was fixed over night with gluteraldehyde at 4°C. The cells were dehydrated using increasing grade of ethanol. The ethanol used was 30%, 50%, 70%, 90% and absolute grade. For each concentration the cells were dehydrated for two hours with one change. Then one drop of cell suspension in absolute ethanol was allowed to dry on small piece (1cm X 1cm) of cut up glass slide. A thin coating of carbon and gold was applied over the cell layer. The gold plated slides were observed under scanning electron microscope at 25 KV accelerating voltage and no tilt.

Antibiogram of *L. monocytogenes isolates*

In the present study, *L. monocytogenes* isolates were tested for their susceptibility to antimicrobial agents by the standard Kirby- Bauer disc diffusion method (Bauer *et al.*, 1966) following National Committee for Clinical Laboratory Standards (NCCLS, 1997) guidelines. All five *L. monocytogenes* isolates were grown in BHI (Brain Heart Infusion) broth overnight at 37°C. The culture suspension was adjusted to 0.5 McFarland Standard (approximately 1.5 x 108 cells). Within 15 minutes after adjusting the turbidity of the inoculums suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times, pressing firmly on the inner wall of the tube above the fluid level to remove excess inoculums from the swab. Mueller-Hinton Agar (Hi-media®) was used as medium to study the susceptibility to antibiotics. Then culture was spread on the entire surface of a dried Muller Hinton agar plate with the sterile culture containing swab. The culture inoculated plate was held at room temperature for 10 minute to allow evaporation of free surface liquid as adopted by Anon (1997).

Commercially available antibiotics octa disks (Hi-Media®) were used: ampicillin (10mcg/disc), Tetracycline (30mcg/disc), Cotrimoxazole (25mcg/disc), Ciprofloxacin (5mcg/disc), Gentamicin (10mcg/disc), Erythromycin (15mcg/disc), Chloramphenicol (30mcg/disc), Cefalexin (30mcg/disc). Ceftrixone (30mcg/disc), Ceftazine (30mcg/disc), Cefotaxime (30mcg/disc), Lincomycin (2mcg/disc), Netilmycin (30mcg/disc), Ofloxacin (2mcg/disc), Vancomycin (30mcg/disc), Amikacin (30mcg/disc) (D0286) Penicillin (10unit/disc), Telecoplanin (30mcg/disc), Clindamycin (2mcg/disc), Ofloxacin (5mcg/disc), Azithromycin (15mcg/disc) were placed on the surface of each inoculated plate using a sterile forceps. After incubation for 24 hours at 37°C, the diameter of the zone around each disc was measured, and interpreted in accordance with the National Committee for Clinical Laboratory Standards (NCCLS, 1997).

Results and Discussions

After two step enrichment procedures using UVM I and UVM II, and also by using Fraser broth streaking is done on PALCAM agar. The growth of *Listeria spp*. in PALCAM agar medium changing the colour of the medium from red to black–

brown or black was ideally obtained in 14 of sample tested. The individual colonies appeared gray green colour with black zone (Fig.1a). The same 14 samples formed yellowish colour (Fig.1b) colonies after streaking on *Listeria* selective agar base. Presumptive colonies were selected for confirmation by streaking on Typtone soya yeast extract agar (Fig.1c). Culture from solid medium incubated at 30- 37^0C were found gram positive (Fig.2a). The organism also cultured in nutrient agar stab that showed characteristics fur tree (Fig.2b) growth due to microaerophilic nature of *Listeria spp*. The organism at 1^{st} and 2^{nd} subculture was more slender compared to those at 4^{th} and 5^{th} subculture. This finding is similar to Todar (2009).

The motility was characteristics of *Listeria spp*. as described by (Roberts *et al*., 2009). The motility was unique not like other motile bacteria as *E. coli* or *Salmonella species*. Variation in temperature has an immediate effect on the peculiar motility. Biochemical test were first done by using Himotility^{TM} biochemical kit for *Listeria* (Himedia®). This kit contains 12 tests. 12 tests were performed by using tentative 14 positive isolates. After performing 12 test as per method mentioned in the protocol, there is found that all 14 isolates were positive for motility + esculin hydrolysis, all isolates were also found to be positive for catalase, methyl red, and Voges Proskauer test, but variation was found in nitrate reduction and sugar fermentation tests (namely xylose, lactose, a- methyl-D- mannoside, Rhamnose, dextrose and mannitol.

In Himotility ^{TM} biochemical kit (Himedia®), *L. monocytogenes* showed positivity for motility + esculin hydrolysis, catalase, methyl red, and Voges Proskauer test, but variation was found in nitrate reduction and sugar fermentation tests namely xylose, lactose, a- methyl-D-mannoside, rhamnose, dextrose and mannitol, mannoside, rhamnose, dextrose and mannitol. Out of 14 isolates 5 isolates fulfill the criteria of *L. monocytogenes*. 5 isolates showed nitrate reduction negative, xylose and mannitol negative, a-methyl-D-mannoside, rhamnose, dextrose positive, and lactose variable, which are the characteristics of *L. monocytogenes* (Fig.3a,b,c). These tests were also done by conventional method (Fig.4).

On the basis of colony morphology in selective media (PALCAM *Listeria* identification Agar Base) and on the basis of gram staining data, 14 suspected isolates were identified as *Listeria* spp. out of 104 tested samples. On the basis of biochemical test and other test like CAMP test and Beta hemolysis, 5 isolates of *L. monocytogenes* were identified out of 14 suspected *Listeria* species. *Listeria monocytogenes* isolates showed CAMP-positivity only with *S. aureus*(Fig.5a) but not with *R. equi* were characterized *as L. monocytogenes* and these five isolates showed a narrow zone of β-haemolysis(Fig.5b).

Data obtained from the above tables shown that the overall prevalence of *Listeria spp.* in unpasteurised raw milk were 14 (13.46%) and *L. monocytogenes* were 5 (4.81%). This finding was almost similar to that of D' Costa *et al.* (2012), where 37 (4.82%) isolates of *L. monocytogenes* from unpasteurised raw milk were isolated. Similarly, Lund *et al.* (1991) reported a prevalence of 3% *L. monocytogenes* from raw milk in his study from dairy farms during a 13- month period (April 1989-April 1990). Gaya *et al.* (1998) also reported a prevalence of 3.62% *L. monocytogenes* from bulk tank 114 farms in Central Spain.

Table.1 Total no. of isolates identified from different sources are given below

Source (milk collected from)	No. of samples examined	No. of positive *Listeria spp*.	% other *Listeria spp.*	No. of positive *L. monocytogenes* isolates	%*L. monocytogenes* isolates
Individual cow's udder	36	3	8.33	1	2.78
Market milk	48	8	16.66	3	6.25
Farm pooled milk	20	3	15	1	5.0
Total	104	1	13.46	5	4.81

Table.2 Antimicrobial drug resistance and Sensitivity pattern of *L. monocytogenes*

Antimicrobial agent	No. of isolates	*L. monocytogenes* isolates from milk					
		Resistant		Intermediate		Sensitive	
		No	%	No.	%	No.	%
Lincomycin	5	2	40	-	-	3	60
Netilmycin	5	1	20	1	20	3	60
Ofloxacin	5	1	20	-	-	4	80
Clindamycin	5	1	20	1	20	3	60
Erythromycin	5	2	40	3	60	0	0
Penicillin	5	0	0	-	-	5	100
Azithromycin	5	4	80	-	-	1	20
Vancomycin	5	1	20	-	-	4	80
Amikacin	5	1	20	1	20	3	60
Ampicillin	5	0	0	2	40	3	60
Tetracycline	5	0	0	-	-	5	100
Cotrimoxazole	5	1	20	3	60	1	20
Gentamicin	5	0	0	-	-	5	100
Ciprofloxacin	5	5	100	-	-	0	0
Erythromycimin	5	3	60	-	-	2	40
Chloramphenicol	5	2	40	3	60	0	0
Ccfalexin	5	1	20	1	20	3	60
Ceftrixone	5	1	20	-	-	4	80
Ceftazidine	5	1	20	-	-	4	80
Cefotaxime	5	1	20	-	40	2	40

Figure.1(a) Growth of *Listeria spp*. on PALCAM agar; (b) growth of *Listeria spp*. on Listeria selective agar; (c)growth of *Listeria spp*. on Typtone soya yeast extract agar

a) b) c)

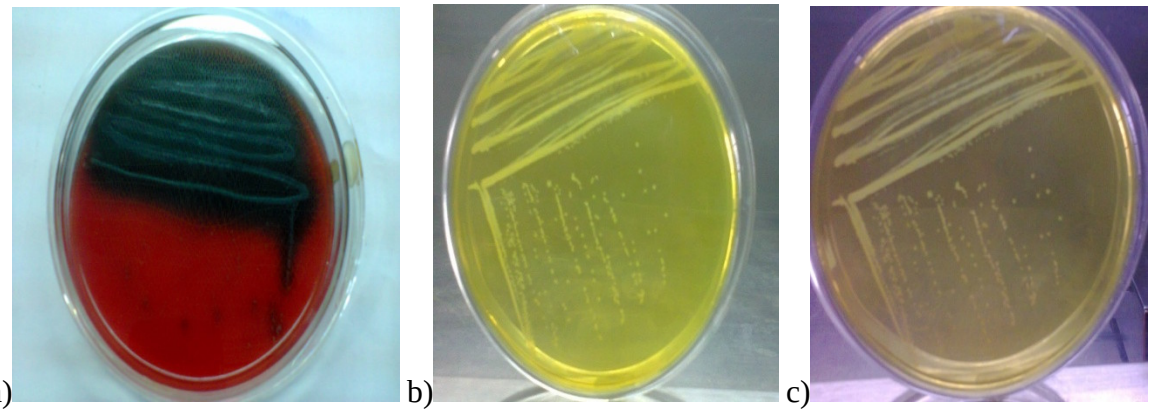


Figure.2(a)Gram staining of *Listeria monocytogenes* (b) fur tree growth of *L. monocytogenes* in nutrient agar stab

a) b)

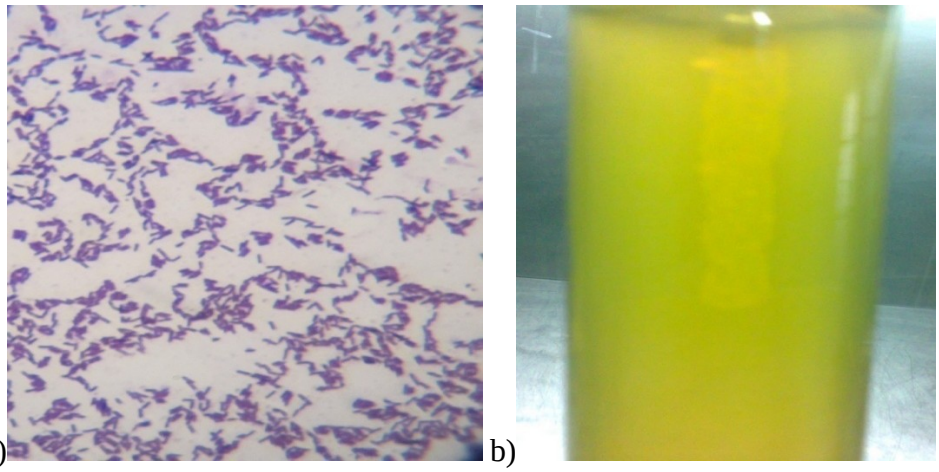
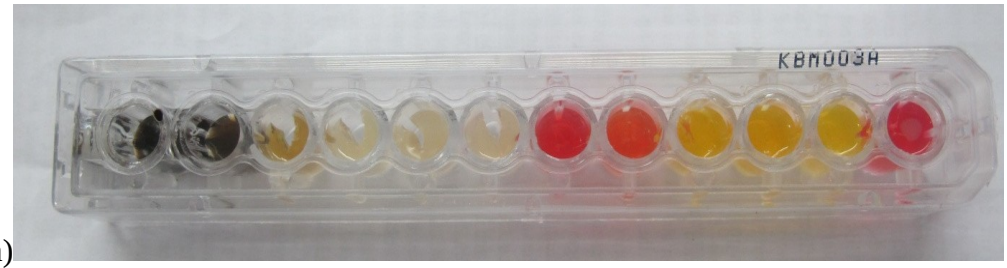


Figure.3 Himotility TM biochemical kit for *Listeria*

a)



b)

c)

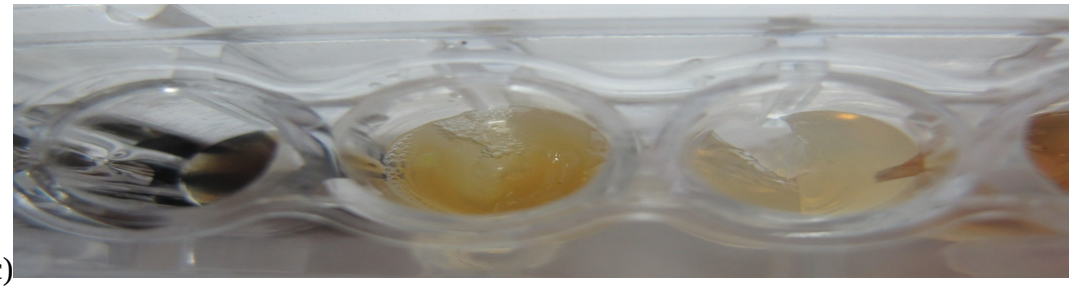
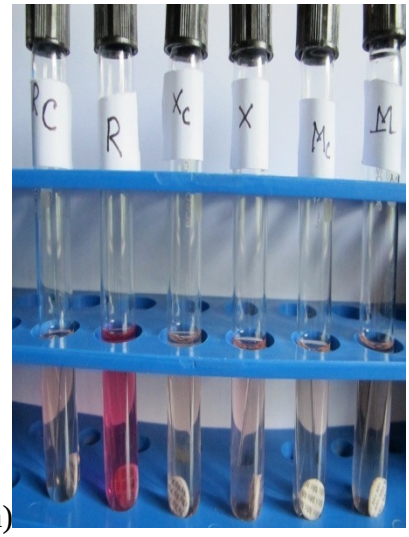
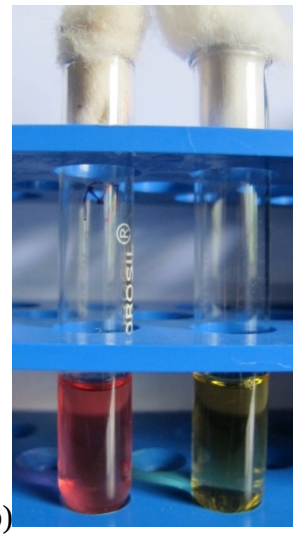


Figure.4 (a) Sugar fermentation test showed positivity for *L. monocytogenes*; (b) Positive Methyl red test; (c) Positive Voges-Proskauer test

(a)



(b)



(c)

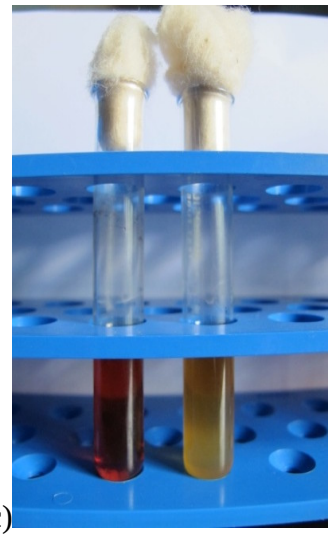


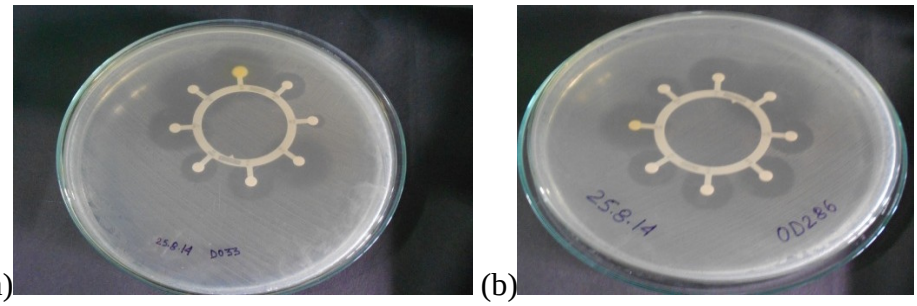
Figure.5 (a) CAMP-positivity only with *S. aureus*; (b)narrow zone of β-haemolysis

(a)

(b)

(c)

Figure.6 Antibiotic sensitivity of *L. monocytogenes* against different antibiotics



Kasalica *et al.* (2011) reported a prevalence of *L. monocytogenes* 2.5–6% in raw milk in Europe. Waghamare *et al.* (2012) reported a prevalence of *L. monocytogenes* 5.88% *in* unpasteurized milk samples from different markets in Mumbai city. Özkan and Boyacıoglu (2004) *reported a* prevalence of *L. monocytogenes* 2% in raw cow milk samples collected in 3 villages in Corum.

The difference prevalence rate of *L. monocytogenes* isolated from milk by different researchers may be due to variation in the geographical region, variation in the size of samples, feeding habit, state of hygiene maintained at the dairy farm level and variation in the storage of milk samples.

The incidence of *L. monocytogenes* isolates were analysed depending on the source of milk collection such as individual cow's udder milk and pooled can milk collected from farm as well as from market by following Chi- square test. In this test it was found that incidence of *L. monocytogenes* were statistically higher in market milk than individual cow's udder milk and farm pooled milk (Snedecor and cochran,1994). All the five *L. monocytogenes* isolates were subjected to antibiotic sensitivity test using Kirby-Bauer disc diffusion method (Bauer *et al.,* 1966). In this method 3 antibiotics, tetracycline, gentamicin, penicillin, exhibited complete sensitivity.

It was found that highest resistant was recorded against Ciprofloxacin (100%), moderate resistant were found against Cotrimoxazole, chloramphenicol and Erythromycin and highest sensitivity was observed against tetracycline, gentamicin and penicillin (100%) (Fig. 6).

The above result were partially correlates with Altuntas *et al.* (2012) who reported susceptibility pattern of *L. monocytogenes* isolates to the antibiotics, such as penicillin G, vancomycin, tetracycline, chloramphenicol, rifampicin, erythromycin, gentamicin and trimethoprim.

The above result partially correlates with Sharif *et al.* (2010) who reported susceptibility pattern of *L. monocytogenes* isolates to gentamicin, doxycycline, ampicillin, tetracycline and penicillinG and resistant to Ciprofloxacin, cotrimoxazole, nalidixic acid and erythromycin. Yu Shu-Bing *et al.* (2004) reported Sensitivity of *L. monocytogenes* to 12 antibiotics including gentamicin, vancomycin, kanamycin, norfloxacin, ofloxacin, erythromycin, chloramphenicol, tetracycline, cephalothin and cefazolin, were carried out. The study revealed that *L. monocytogenes* was resistant to enrofloxacin and nitrofurantion. Enurah *et al.* (2013) reported Chloramphenicol was the most effective

antibiotic against the *L. monocytogenes* isolates with the least resistance (3.70%) while nalidixic acid proved to be least effective with resistance of 90.74%.

This study was undertaken to estimate the prevalence of *L. monocytogenes* presents in raw milk samples in and around Kolkata, west Bengal. In this study, 13.46%samples were found to be positive for different *Listeria spp*. and 4.81% samples for *L. monocytogenes*. In antibiotic sensitivity assay, *L. monocytogenes* isolates shown highly sensitivity towards to tetracycline, gentamicin, penicillin resistance, moderately sensitive to ampicillin, vancomycin and resistant to ciprofloxacin (100%).

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