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Prevalence and antimicrobial resistance of Listeria monocytogenes isolated from raw milk and dairy products

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ABSTRACT

Listeriosis is a severe foodborne disease commonly caused by eating contaminated food with the Listeria species. A large variety of foods, especially dairy foods and ready-to-eat products, can support the growth of pathogens. Outbreaks of listeriosis have been related with milk, cheese, vegetable salads, and meat products, and fatality rates are typically around 20% due to listeriosis. The Centers for Disease Control and Prevention evaluates that 2500 infections and more than 500 deaths are related with listeriosis each year in the United States. A total 125 milk and dairy products were included in the study. Isolation and identification of this specie was done and then confirmed it by gram staining. Antimicrobial sensitivity was also checked. Prevalence of Listeria species were 16.8%, Listeria monocytogenes was 13.6%. Listeria monocytogenes was resistant against Ampicillin, Amoxicillin, Penicillin and sensitive Fosfomycin, Ciprofloxacin, Gentamycin. The results of this study showed the low prevalence of Listeria monocytogenes.

1. Introduction

Listeriosis is a severe foodborne disease commonly caused by eating food contaminated with the Listeria species. A large variety of foods, especially dairy foods and ready-to-eat products, can support the growth of various species of Listeria. Outbreaks of listeriosis, have been related with milk, cheese, vegetable salads, and meat products with fatality rates around 20%. The Centers for Disease Control and Prevention evaluated that 2500 infections and more than 500 deaths are related with listeriosis each year in the United States (Huang et al., 2015). Listeriosis caused by L. monocytogenes can be differentiated into two types; non-invasive gastrointestinal listeriosis and invasive listeriosis. Development of antimicrobial resistance among bacterial pathogens is another test for human wellbeing. By knowing antimicrobial susceptibility pattern, can use antimicrobial drug more wisely. (Roberts et al., 2009). The susceptibilities of various microorganisms are checked against different antimicrobial agents by Kirby-Bauer disc diffusion method as institutionalized by the National Committee for Clinical Laboratory Standards. (Morobe et al., 2009). In the dairy industry, directly and indirectly Listeria contaminate the food, food products, environment and raw milk resulting in the destruction of the economy and public health. Medical and scientific groups are focused on the safety and security of all these products (El Marnissi et al., 2013). Previously susceptible L. monocytogenes become resistant to antimicrobial drugs currently in use for both human and veterinary medicine. Currently, there is inadequate information regarding the prevalence and antimicrobial susceptibility patterns of Listeria spp. in foods. Therefore, present study was undertaken to determine the prevalence and antimicrobial resistance profiles of Listeria monocytogenes isolated from raw milk and milk products samples collected from District Faisalabad.

2. Materials and methods

2.2. Study Area and Sampling:

A total of 125 Milk and milk product (yogurt and cheese) samples were collected from the different areas of District Faisalabad. Number of samples required was calculated as follows
(Crépet et al., 2007).

$$n=([\![1.96]\!]^2*P\_exp\ (1-P\_exp))/d^2$$

Where, Pexp is prevalence expected and d2 is desired absolute precision. With expected prevalence of 9% (Chandio et al., 2007) and desired absolute precision of 5%, calculated number of samples as under.

$$n=([\![1.96]\!]^2*0.09(1-0.09))/[\![0.05]\!]^2 = 125$$

2.3. Isolation of Listeria species:

Samples were placed at 4oC for two days. Because Listeria monocytogenes grows better at low temperature than other species of bacteria, the process is known as cold enrichment. (Al-Mariri et al., 2013).

2.4. Cultivation and Identification of Bacteria:

Oxford Listeria selective agar was used for the growth of Listeria monocytogenes. Listeria agar base have a specific composition (oxford formulation).

Table 1 Composition of Oxford Listeria Selective Agar

Compounds	Composition
Columbia Blood Agar	39.0 g
Acsuline	1.0 g
Ferric Ammonium	0.5g
Lithium chloride	15.0g

Supplements were also added to the Listeria agar base. These supplements are selective for the listeria.

Table 2 Listeria Selective Supplement (oxford formulation SR0140E)

Compounds	Composition
Cycloheximide	200.0 mg
Colistin Sulphate	10.0 mg
Acriflavin	2.5 mg
Cefotetan	1.0 mg
Fosfomycin	5.0 mg

2.4. Growth on blood agar:

Presumptive positive samples were inoculated on blood agar and incubated for 24 hours at 37oC to observe hemolysis pattern. (Maijala et al., 2001).

2.5. Confirmation of isolates:
Bacterial isolates were confirmed by morphological and biochemical characteristics. Gram staining was carried out to observe their Gram reaction (Davies et al.,1983 & Beveridge and Davies, 1983).

2.6. Biochemical Tests:
Different biochemical tests including catalase, methyl red test, oxidase etc. were performed for the confirmation of Listeria monocytogenes (Aygun and Pehlivanlar, 2006).

2.7. Antimicrobial Susceptibility Test:
Antimicrobial susceptibility test was performed on the basis of Kirby Bauer disk diffusion method (Tiwari et al, 2011).

2.8. Statistical Analysis:
The data obtained in this study was analyzed by two factor ANOVA.

2.9. Results

125 milk and milk product samples were collected from the different areas of faisalabad by using that formula which is shown in methodology.

Table 3 Total number of sample collection (Raw Milk, Yogurt and cheese)

	Samples	Size
1.	Raw Milk	75
2.	Cottages cheese	25
3.	Yogurt	25
	Total	125

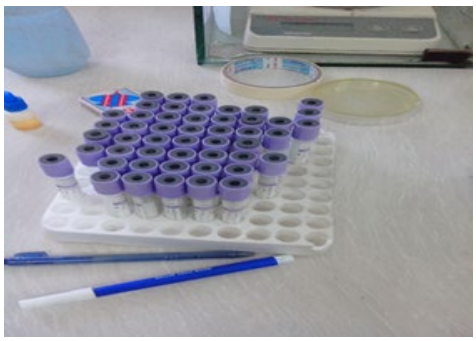

Fig. 1 Sample collection

Out of 125 samples of raw milk and milk products, 21 samples were positive for Listeria spp. From these positive samples, 17 were positive for Listeria monocytogenes. 75 raw milk samples were tested in which 12 samples were positive for listeria monocytogenes. 25 samples of each yogurt and cottage cheese were included in the study that yielded 2 and 3 cultures were positive for Listeria monocytogenes, respectively. Raw milk showed highest percentage positivity of Listeria monocytogenes recorded as 16% followed by cheese (12 %) and yogurt (8%). Results of positive numbers of test samples are summarized in table 4. % age positivity of listeria monocytogenes for tested samples are shown in fig. 2.

Table 4 Positive samples of Listeria species

Sample Name	Sample size	Positive	*Listeria monocytogenes*	Other Spp.
Raw milk	75	16	12	4
Yogurt	25	2	2	0
Cottage Cheese	25	3	3	0
Total	125	21	17	4
%age		16.3	13.6	3.2

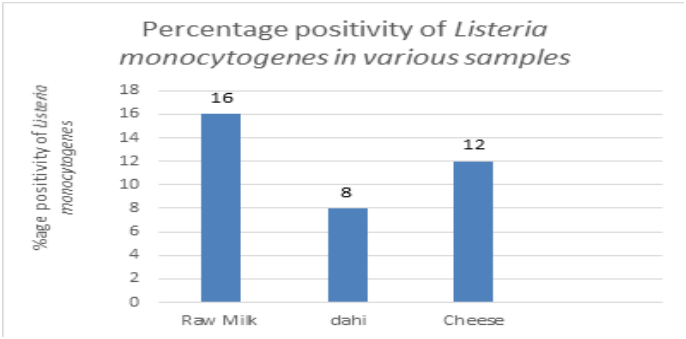

Fig. 2 Percentage positivity of Listeria monocytogenes in various samples

3. Isolation of Listeria Species
3.1. Media pouring
Media was poured in different petri plates.

Fig. 3 Oxford Listeria Specific Agar pouring

3.2. Labelling the plates:
Labelled all petri plates for streaking and streaked the four sample at one petri plates.

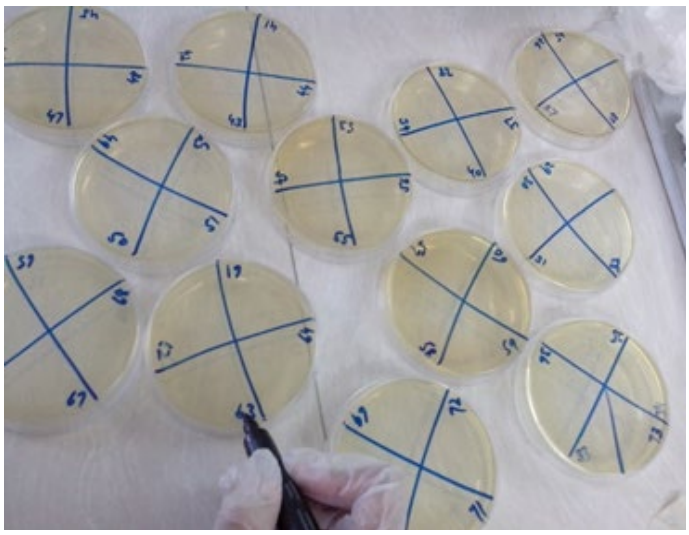

Fig. 4 Labelling of agar plates

3.3. Streaking
After the labelling, streaking was done.

Fig. 5 Streaking of samples on selective agar

4. Incubation period:
When the streaking was done, then put all the plates in the incubator at 36OC for 24 to 48 hours.

Growth observation: After the incubation period, saw some colonies on the petri plates, but some plates have no growth.

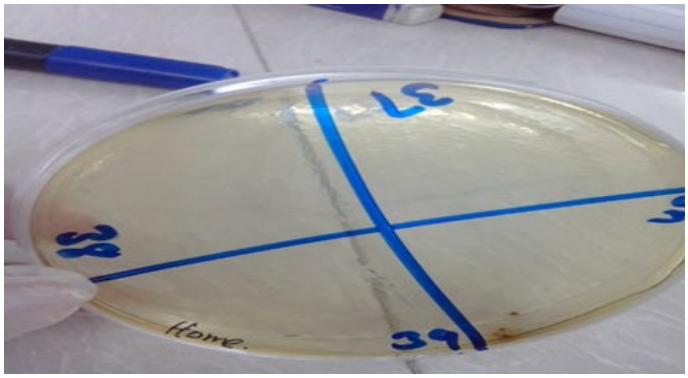

Fig. 6 No growth on this plates (shows negative result)

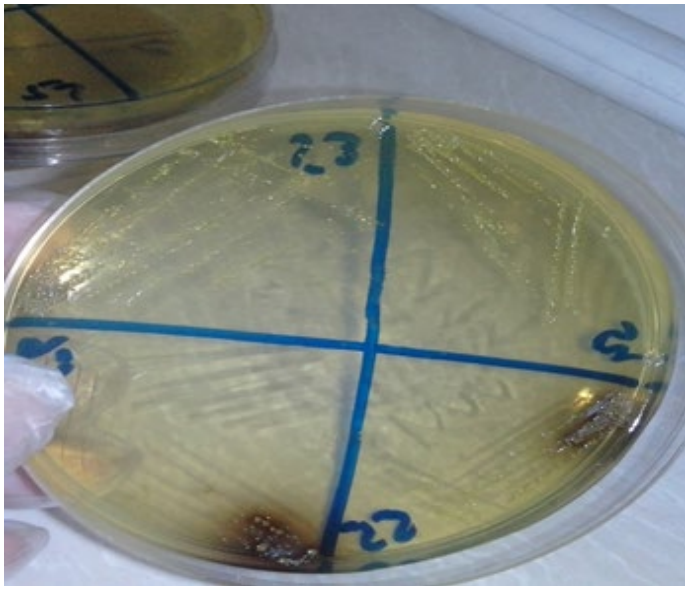

Fig. 7 Growth of bacteria on Oxford Listeria Specific Agar (Shows positive result)

5. Growth on blood agar:
Suspected colonies inoculated on blood agar and incubated for 24 hours at 37oC, to observe the hemolysis pattern. B hemolysis was observed for listeria monocytogenes often produce only narrow zones of hemolysis that frequently do not extend much beyond the edge of the colonies

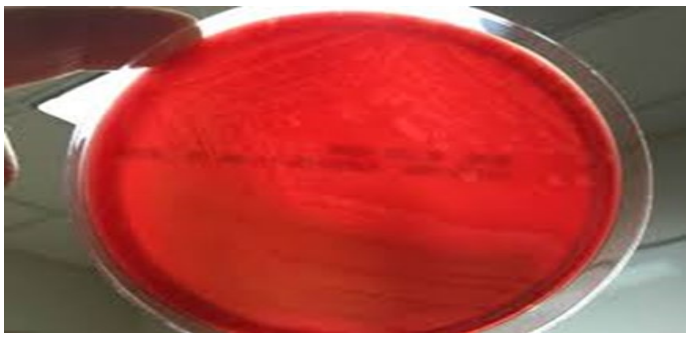

Fig. 8 Hemolysis on blood agar

6. Incubation period:
Bacterial isolated were confirmed by morphological and biochemical characteristics. Gram staining was done to observe their Gram reaction.

Table 5 Principle characteristics of Listeria species

Test	*Listeria* spp. Reaction
Gram stain	Positive
Cell morphology	Short (0.4-0.5 μm × 0.5-2.0 μm) nonspore forming rod with a few peritrichous flagella
Growth conditions	Aerobic and facultative anaerobic
Motility	Positive tumbling motility at 25°C, negative at 37°C

7. Gram Staining:

Fig. 9 Staining of bacteria shows short rods, purple color

8. Biochemical Tests:
Different biochemical tests were performed for the identification of Listeria monocytogenes including Methyl Red tests, catalase and oxidase.

9. Catalase test:
Catalase test was positive for the listeria monocytogenes.

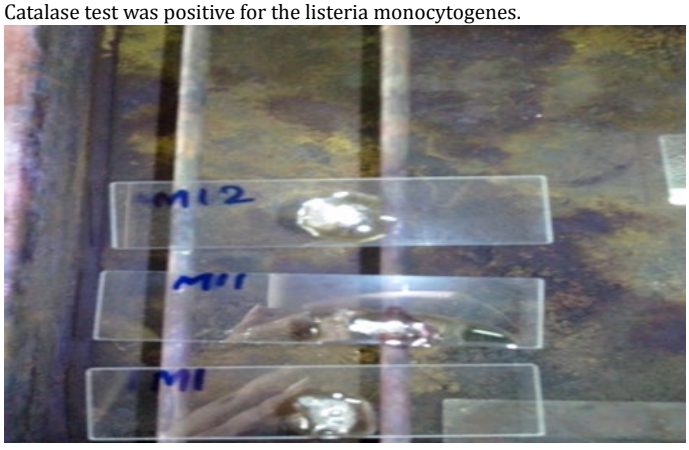

Fig. 10 Catalase test

10. Methyl Red Test:
PMR was positive for Listeria monocytogenes. Red color shows the positive result.

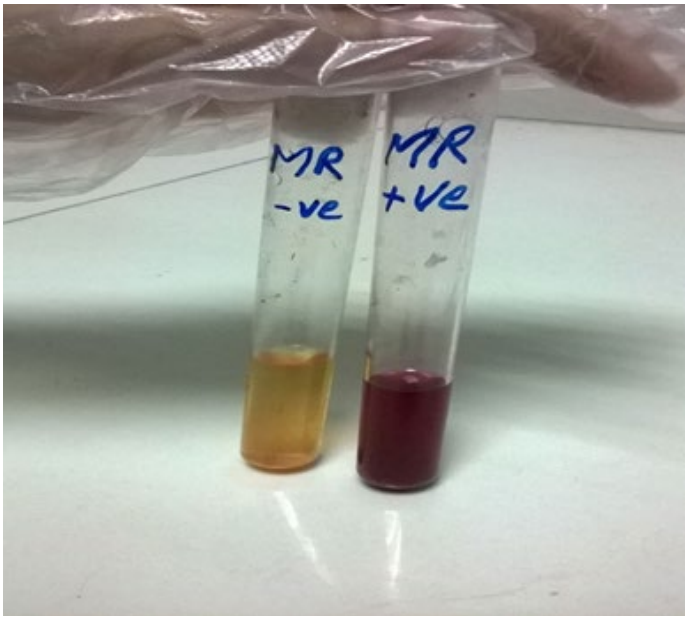

Fig. 11 Methyl Red test

Table 6 Results of the Biochemical test

Biochemical test	Results
Catalase	Positive
Methyl Red	Positive
Oxidase	Negative
Gram Staining	Positive

11. Antimicrobial Susceptibility test:
Kirby-Bauer disc diffusion method was used to examine the antimicrobial susceptibility patterns against commonly used antibiotics. Antibiotics which were used that's mention in the methodology. Antibiotic susceptibility profile. Antibiotic susceptibility patterns of all the confirmed Listeria isolates were performed by standard disc diffusion method as described earlier. Antibiotic discs used were penicillin, ciprofloxacin, ampicillin, fosfomycin, amoxicillin and gentamycin (Oxoid). Assessment of antimicrobial susceptibility profile of L. monocytogenes revealed the presence of four multi-drug resistant isolates. The higher resistance rate was recorded for penicillin, ampicillin and amocillin. All L. monocytogenes identified in the current study were sensitive to ciprofloxacin, gentamicin and fosfomycin.

Table 7 The sensitivity and resistance against specific antibiotics

Antibiotics	Resistant	Sensitivity
Ampicillin	83.37%	-
Fosfomycin	-	87.8%
Ciprofloxacin	-	98.68%
Amoxicillin	96.25%	-
Penicillin	98.5%	-
Gentamycin	-	100%

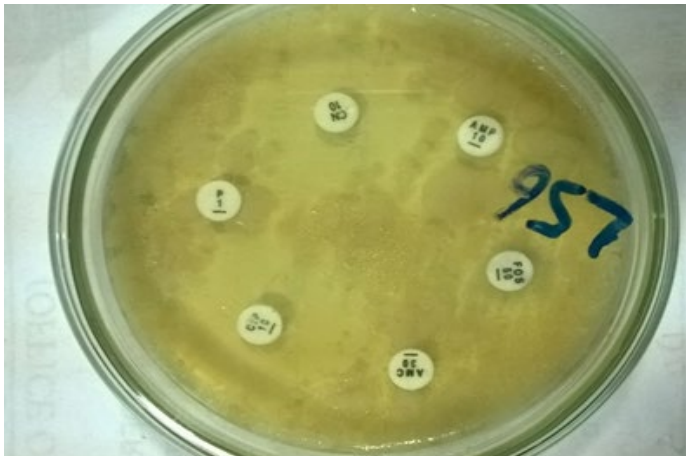

Fig. 12 The resistance of antibiotics

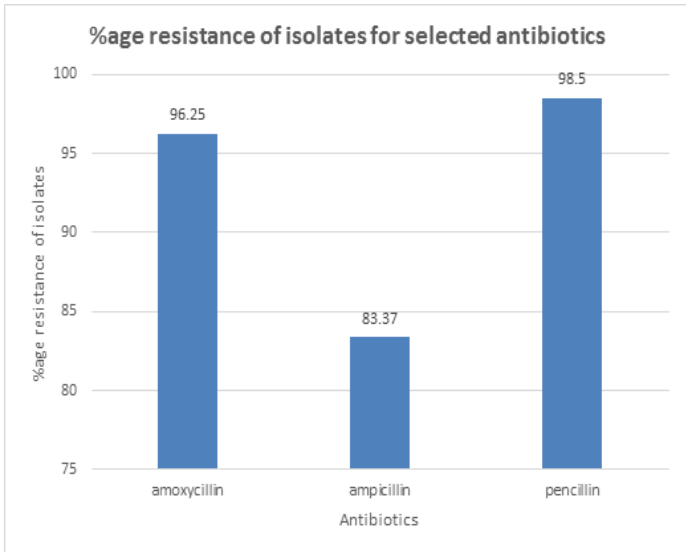

Fig.13 The resistance of antibiotics percentage

This graph shows the % age resistance of Listeria monocytogenes against antibiotics amoxicillin, ampicillin and penicillin.

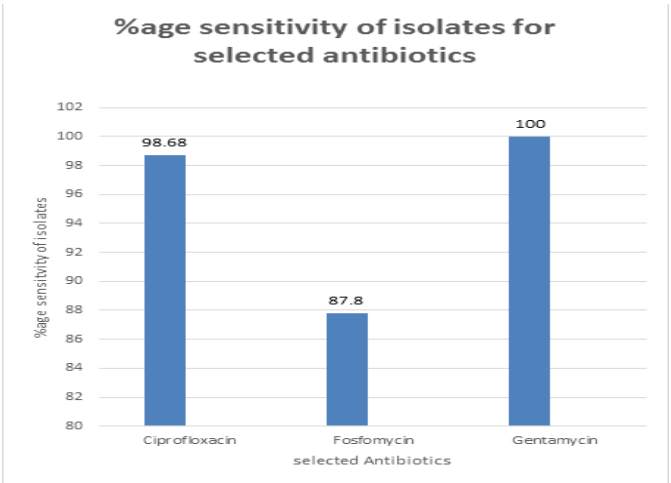

Fig. 14 Percentage sensitivity of isolates

This graph shows the sensitivity of Listeria monocytogenes against specific antibiotics ciprofloxacin, fosfomycin and gentamycin.

Discussion
Diseases are caused by many pathogenic bacteria which is present in milk, these are also the main factor in public health. Diseases which are caused by listeria monocytogenes is called listeriosis. Listeriosis is caused by using raw milk, milk products and other food. In this studied different samples of

braw milk, cheese and yogurt collected from the different area of the Faisalabad. 300 samples in the USA were collected in which 9(3 %) was positive for Listeria monocytogenes. Soyutemiz E et al., (2001) collected 100 samples of milk for the find out the prevalence of listeria monocytogenes was reported 3% in Turkey. The same study was carried out in Spain in which 67 samples, but the prevalence rate 44.7 % very high for listeria monocytogenes by Garayzabal et al., (1987). In USA, another study was carried out by Lovett et al., (1987) which showed high occurrence of listeria monocytogenes because 200 samples were observed in which 14 were positive for listeria monocytogenes. Harvey et al., (1992) studied 176 samples in which prevalence is 15.3 % for Listeria monocytogenes. Listeria is present in the surrounding, mostly present in contaminated milk due to unhygienic conditions. Milk is contaminated during handling, storage and processing.

Mahmood et al., (2001) collected 400 samples of milk in Faisalabad and when study was carried out there is 7.5% of listeria species were isolated in which 2.25% of listeria monocytogenes. Here, the prevalence rate of this study is very low as compare to this study results which was 13.6%. Jamali et al., 2013 study showed some Listeria monocytogenes the isolates were resistant to ampicillin, erythromycin, amoxycillin, penicillin, chloramphenicol, oxytetracycline, vancomycin and tetracycline. Results of this study were similar with study. The maximum occurrence of the resistance was for tetracycline which was 34.6% (Charpentier et al., 1995). Although most researchers have reported that strains of Listeria spp. are not resistant to vancomycin. Our results agree with those of Conter et al., (2009) and Harakeh et al., (2009) who also found Listeria spp. isolates resistant to vancomycin in their studies. Because of the transferable elements between organisms, resistance rapidly becomes more common. In numerous studies transfer by plasmids conjugation, and the transposons carrying some antibiotic genes from the other bacteria to Listeria and between listeria species have been reported. Moreover, using the antibiotics for prophylaxis and as development stimulants is major reason for some antibiotic resistance.

Aslantas and Yildiz, 2003 study showed that the only food industry and the food laboratories and food industries related with listeriosis. Western countries eat different as compare to eastern countries. The majority of people prefers to consume traditionally produced foods. Furthermore, most cattle and sheep farms in Turkey does not have adequate hygiene precautions and animals live in a natural environment together with people. Ozbey et al., 2006 aimed to study was determine the occurrence of Listeria spp. in food, the environment, in animals and to observe the relationship between these parameters in east. The occurrence of Listeria spp. was found to be in feed was 2.3 % but water, environment, faeces and raw milk samples in sheep farm houses was 9.4%. Approximately half of these isolates were found to be pathogenic for sheep and cattle and other remaining were pathogens present in all humans and animals. A large number of studies have reported on the presence of Listeria in various animals and foods. The occurrence of Listeria described in Turkey is related to the occurrence reported internationally (Abay and Aydin, 2005).

Ertas and Seker, (2005) study showed the highest resistance was observed against nalidixic acid, penicillin and erythromycin 100 % tested Listeria species, ampicillin was 83.33 %, cephalosporin was 60.26 %, trimethoprim was 67.95 %, nitrofurantoin was 64.10 %. This study results were closely related to this study in which pencillin, ampicillin, amoxicillin was resistance to listeria monocytogenes but fosfomycin, gentamycin and ciprofloxacin was sensitive to this organism.

Conclusion

The results of this study showed the low prevalence of Listeria monocytogenes. However, listeriosis is present in world which is a very hazardous disease, so this low incidence of Listeria monocytogenes can't be ignored. Raw milk and milk products also responsible for the listeriosis. By understanding all these problems/conditions, suggested some improvement in milk handling, processing and production technology. Consumers should also take care for the prevention of pathogens.

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