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Original Article

Status of *Listeria monocytogenes* in Pooled Milk and Milk Product in and Around the Area of Addis Ababa, Ethiopia

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Abstract

Despite advances in food science and technology, food borne disease remains one of the major public health problems all over the world and particularly causing a massive health tragedy in the developing countries. *Listeria* is one of the important emerging zoonotic diseases affecting animal and human health following the consumption of contaminated food of animal origin. Predominantly the ability of *Listeria* to survive in extreme condition and cause food contamination has become a major concern. This study was conducted between November 2021 to April 2022 on pooled milk and other milk products in and around the area of Addis Ababa to provide insight in to the method of isolation and identification of *Listeria from* pooled milk sample and other milk products found on different market places. A total of 180 samples consisting of pooled milk, yogurt and cheese samples were collected. The samples were examined by the conventional techniques set by International Organization for Standardization and world organization of animal health using fully automated coated micro plate (MALDI-TOF) based bacterial identification system, third generation micro plate for confirmation and sub species identification. From the total of 180 samples, 23(6.0%) of the samples were presumptively positive for *Listeria* by conventional biochemical tests. However, the presumptively positive samples were found to be negative when they subjected to the biolog identification system. The biolog identified those presumptive samples as another bacterial species that is characteristically similar with *Listeria* in biochemical tests. Thus, the isolation and identification of *Listeria* in this study found to be zero. Even if, absent of *Listeria* in this study may not fully indicate the absence of *Listeria* in the study area, we dare to question the reliability of *Listeria* identification findings where isolation was solely done with biochemical tests. Henceforward, isolation and identification of *Listeria* organism should not be reached to the conclusion only by relying on biochemical test, whilst there are other bacteria characteristically similar with *Listeria*.

Keywords: Addis ababa; MALDI-TOF; *Listeria*; Milk; Milk product

Introduction

Food borne diseases mostly affects developing countries, such as Ethiopia due to major contributing factors such as overcrowding, poverty, changes in eating, habits, mass catering, complex and lengthy food supply procedures with increased international movement, inadequate sanitary conditions, poor general hygiene practices and besides the common factor in Ethiopia the wide spread habit of raw milk and traditionally prepared yogurt is a potential cause for food borne illnesses [1]. Over 2 million people die each year of pathogens borne from food in developing countries [2].

Among pathogens associated with food borne outbreaks; *Salmonella, Escherichia coli O157:H7, Campylobacter, and Listeria monocytogenes* are responsible for the majority of outbreaks [3]. More than 110 outbreaks worldwide have been reported including the largest one in South Africa in 2018. Although outbreaks have been reported from several countries, the majorities of human cases are sporadic and represent a real challenge to controlling them definitively. Possible explanations for the emergence of human food-borne *Listeriosis* as a major public health concern include major changes in agricultural methods and animal husbandry, food production, processing and distribution, increased use of refrigeration as a primary preservation means for foods, changes in human eating habits, particularly towards convenience and ready-to- eat foods, and an increase in the number of people considered to be at high risk for the disease (elderly, pregnant women, newborns, immunocompromised) [4]. If *L. monocytogenes* has been reported in several countries, its incidence depends on eating habits, cooking practices, use of refrigeration and food importation. *Listeriosis* is a global problem as it adversely affects animal health, quality of milk and the economics of milk production, affecting every country, including developed ones and causes huge financial losses [5].

Listeriosis is an important emerging zoonotic disease affecting human health following the consumption of contaminated foods of animal origin. Among the different species of the genus *Listeria*, *L. monocytogenes* is the causative agent of *Listeriosis*. [6]. *L.*

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monocytogenes can persist for long periods in the environment or as an asymptomatic infection in adult animals and birds due to its psychrophilic nature, it can grow within a wide range of temperatures (-1.5 to 50ºC) [7]. *L. monocytogenes* can persist for long periods in the environment and can easily contaminate agricultural products and ultimately livestock products. *L. monocytogenes* is an important cause of diseases in both animals and humans. In the vast majority of human cases, infection is the result of consumption of contaminated food. [8]. Finally, hypervirulent *L. monocytogenes* clones have adapted to mammalian gut, which accounts for their association with dairy products [7].

Reports have indicated that *Listeria spp*. including *L. monocytogenes* is most frequently prevalent in the milk-processing environment including steps, drains and floors. In addition, different studies reported that a higher prevalence of *Listeria species* was found milk samples and also other milk products [9].

The occurrence of *Listeriosis* among humans has received increasing attention as epidemic *Listeriosis* has been recognized and reported in immunosuppressed populations. The bacterium principally causes intrauterine infection, meningitis and septicemia [10]. *Listeriosis i*n pregnancy may be asymptomatic or manifest as severe systemic infection in the unborn or newly delivered infants [11]. The microorganism causes fatal infections such as encephalitis, sepsis and meningitis in immune deficient patients and abortion in pregnant women. The mortality rate is reported to be 20–30% *Listeria spp*. are widely distributed in the rural environment and may in this manner contaminate milk and production plants [12].

Moreover, it was reported that cattle farms play a bigger role in the spread of *Listeria* between animals or people rather than small ruminant farms [13]. Ruminant farm animals play a key role in the persistence of *Listeria spp*. in the rural environment via a continuous fecal-oral cycle [9]. The risk of *Listeriosis* in ruminants increases with poor quality fermented feeds, for example, when dairy cattle are fed with ensilage foods [14]. Furthermore, *L. monocytogenes* may also contaminate milk from animals with. In addition to the quality of silage, other hygiene parameters, ensured by good herd health management, contribute heavily to the microbiological quality of the milk [15].

Statement of the Problem

Beside the limitation of published resource in Ethiopia about the prevalence of *Listeria* there have been some reports on its occurrence and distribution in retailed meat and milk product at similar study area with the current study [16]. Although outbreaks have been reported from several countries, the majorities of human cases are sporadic and represent a real challenge to controlling them definitively. Possible explanations for the emergence of human food-borne *Listeriosis* as a major public health concern include major changes in agricultural methods and animal husbandry, food production, processing and distribution, increased use of refrigeration as a primary preservation means for foods, changes in human eating habits, particularly towards convenience and ready-to-eat foods, and an increase in the number of people considered to be at high risk for the disease (elderly, pregnant women, newborns, immunocompromised) [4].

On the other hand, on human health medical school studies done in Ethiopia on the issue there are reports about higher rate of abortion because of *L. monocytogen*. The bacteria were isolated in 8 (5.56%) of 144 screened women. The isolation rate of *L. monocyogen* was relatively higher among women with a history of fetal loss (9.7%), followed by women with preterm delivery (6.25%). One of the six cord blood was positive for *L. monocytogen*, indicating that the Trans placental transmission rate was 16.7%. More than 2% of women with an ongoing pregnancy were found to have *L. monocytogen* septicemia, which could hurt their fetus [17]. Pregnancy- related *Listeriosis* is an important public health concern of feto-maternal units due to the high morbidity and mortality to the fetus and or mother [18].

Basically, this paper attempt to find out the bacterial occurrence on milk and milk products. Because of the bacterial character of becoming a treat to the public health especially for pregnant women and animal health with its high character of resistance to most of environmental conditions clearly shows the need for further researches.

Objective of Study

- To know the status *Listeria spp* in pooed milk and milk products in and around Addis Ababa.

Materials and Methods

Study Site

The study will conduct in Addis Ababa and the cities found around Addis Ababa. Addis Ababa is the capital city of Ethiopia and African Union located at 9.1.1.48N & 38.44,.24E which is located at 8°30'and 40°27' N latitude and 34°21' and 39°1' E longitude. Also include areas around of Addis Ababa. Addis Ababa lies at an area of 2, 355 meters [19] (Figure 1).

Study Population

Lactating cow find in Addis Ababa dairy farms and other intensively build farm around the city of both breeds namely cross breed (Holstein Friesian-zebu crosses) and local zebu breed were including during the study period. Also, milk products that are the product of combination of different milk extracts found in a market place which also include sample from pasteurized milk and other milk extract like yogurt and cheese were collected to isolate and identify *Listeria* in pooled milk.

Study Design, Sampling Techniques and Sample Size Determination

A cross sectional study was conducted between the periods November 2021 to April 2022. The sampling technique we used were purposive sampling technique, which based on willingness of farm owners and availability of culture media for the isolation of *Listeria* organism. A total of 180 samples were collected from different farms in the study areas and transported to laboratory for isolation.

Study Protocol Design and Method

Iisolation and identification of ***Listeria*****:** The isolation and identification of *L. monocytogenes* from samples from the food chain and specimens from animal require the use of selective agents and enrichment procedures that keep the levels of competing microorganisms to reasonable numbers and allow for the multiplication of *L. monocytogenes* to levels that are enough

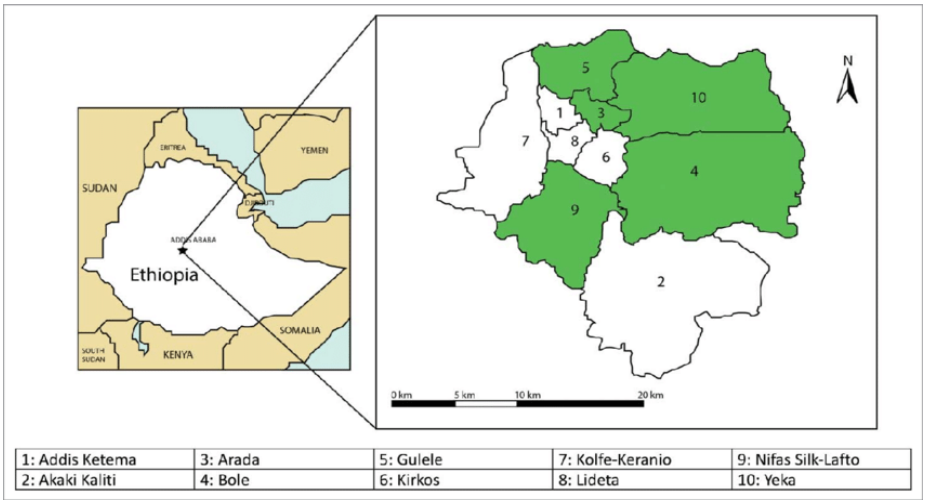

Figure 1: Study area in and around Addis Ababa.

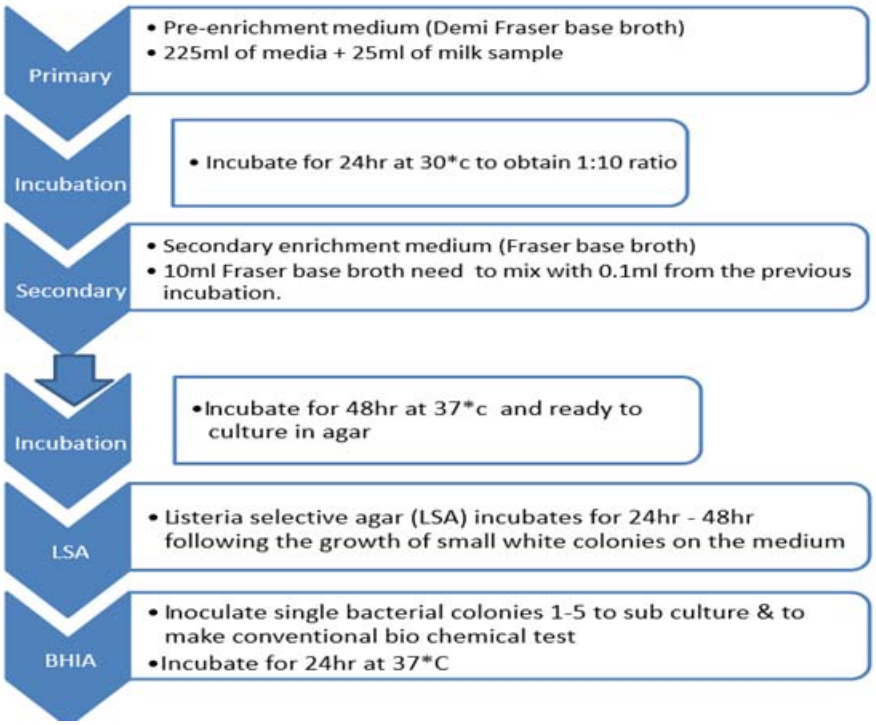

Figure 2: Chart of *Listeria* isolation procedures.

for detection of the organism. In the early days of *Listeria* clinical bacteriology, cold enrichment broths were regularly used to this end [6].

The primary enrichment step involved a selective liquid median with reduced concentration of selective agents and the medium is known as half Fraser broth. The half Fraser broth enrichment medium 3M MEDIA, Ltd., Basingstoke, UK, CM0895. Contained net volume of lithium chloride (3g/10ml of distil water) and half volume of both acriflavine hydrochloride (0.25g/100ml distilled water) and sodium salt of nalidixic acid (0.1g/10ml sodium hydroxide solution) (half Fraser broth). Raw milk and other milk products samples were tested for the presence of *Listeria spp* following the procedure recommended by using the International Organization for Standardization [20]. A 25 g representative portion from each sample was introduced aseptically into a sterile 1000ml bottle containing 225 mL of Half Fraser Broth (3M MEDIA, Ltd., Basingstoke, UK, CM0895) (primary enrichment medium) to obtain a 1:10 followed by incubation for 24 h at 30°C [21].

The secondary enrichment medium Fraser broth (3M MEDIA, CM0895) with full concentration of selective agent was employed. After incubation period of the primary enrichment broth, 0.1 mL sub-sample from each Half Fraser Broth culture was added to 10

mL of Fraser Broth (3M MEDIA, CM0895) (secondary enrichment medium), and incubated for 48 h at 37°C. A loopful of the Fraser Broth enrichment culture streaked on the surface of *Listeria* selective Agar (TM MEDIA, CM1084) [22].

Listeria selective agar consist of essential nutrients which enhance growth of *Listeria* selectively from those peptic digests of animal tissue (10g/l) and thiaminium dichloride (0.0005g/l) vitamin B for growth improvement of *Listeria*, thiocyanate and nalidixic acid (0.040g/l) inhabit growth of gram-negative bacteria and acriflavine hydrochloride (trypaflavine 0.010g/l) recommended for *Listeria* growth also support growth of other gram negative and positive bacteria [20]. These selective agars were then incubated for up to 48hr at 37°C. Selective agars were observed for suspected colonies at 24hr and 48hr of incubation. Suspected colonies were those that appeared white and small sized colonies. Whenever possible, up to 5 suspected colonies showing typical morphology of *Listeriae* on these isolation media were streaked onto brain heart infuse agar (Oxoid, M290) and incubated at 37°C for 24h. The following tests were used for confirmation; Gram's staining, motility test, catalase reaction [23] (Figure 2).

Biochemical test:

Morphological observation: Colonies of bacteria in a petri dish were observed for color, nature, edge, elevation, surface, and size of bacteria.

Gram staining: Bacteria to be colored were taken and a fixed loop was formed over the object glass; this was dripping with crystal violet and was subsequently allowed to stand for 1-2 minutes before being washed with distilled water. Then, iodine solution was used and the solution was allowed to stand for 1-2 minutes before being washed with 95% alcohol to clean the sample, followed by distilled water. The mixture was given safranin and allowed to stand for 1-2 minutes before being washed with distilled water and then dried. Preparations were examined under a microscope to assess the structure, morphology and color of the cells of the bacteria.

MALDI TOF for confirmatory test: The application of matrix assisted laser desorption/ionization-time-of-flight (MALDI-TOF) as a single identification and source-tracking tool for a collection of *Listeria species* isolates, obtained predominantly from dairy sources will be explored. The isolates are going to be cultured on different growth media and analyzed using MALDI-TOF [24]. The two incubation times (24 and 48h) are used Whilst reliable genus-level identification will be achieved from most media, identification at the species level was found to be dependent on culture conditions protein (p60) is a surface cell wall protein of *Listeria species* and it is released in the medium of culture. It is responsible for the successful invasion of host cells [25].

Questionnaire Survey

A pre-tested structured questionnaire was developed and information regarding animal data like breed, age, lactation length, milk yield, previous history, presence lesion and management aspects like herd size, milking practice and milk handling method, before milking and after milking data were collected Parker (2009). At sub-clinical bases asking the owners about previous history of abortion and any sign of circling disease in their previous experience to identify *Listeria* [26].

Statistical Analysis

The collected data were first entered and managed into Microsoft Excel worksheet and analyzed by statistical software, namely, SPSS version 22. Prevalence was determined by the formula described by [27]. and calculated as the rate of number of diseased animals and total number of animals in a given dairy farm. Associations between explanatory variables (species of animals, age, and health status) and questioner's survey were done by chi-square test, and $P < 0.05$ was set to indicate significance. By using the following formula.

$$N = \frac{1.96^{2*} Pexp^{*} (1 - Pexp)}{d^2}$$

Where: N = required sample size,

1.96= the value of Z at 95% confidence interval

Pexp = expected prevalence (50%)

d = desired absolute precision (5%)

Results

From the 180 sample which consist of 80 pooled milk sample from farms found in and around Addis Ababa and also 50 sample of cheese from market places that most people of the city will reach and 50 sample of yogurt from fifty shop which sell milk products and also pasteurized milk of some companies are also examined in the isolation and identification process of *Listeria* (Table 1).

Conventional Bio-chemical Test

Morphological identification: Based on the results of the study of morphological characteristics and *Listeria monosytogenes* colonies adapted to have a white encircled colonial structure [28]. Members of the *Listeria* genus are short rods, facultative anaerobic, Gram positive, not forming spores and capsules, distributed individually, and in form of short chains and small white colony on LSA agar (Figure 3),

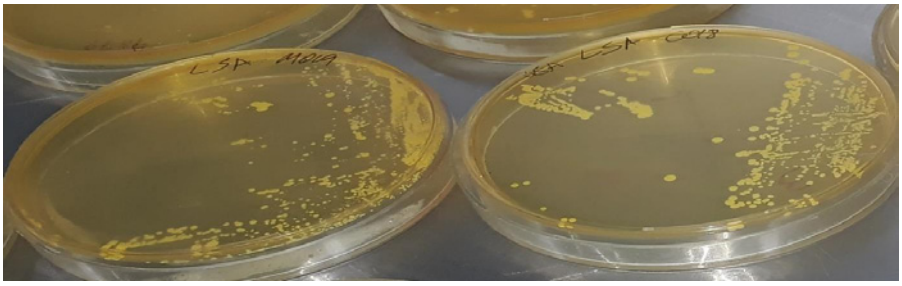

Figure 3: Morphological characteristic of *listeria* suspected isolate on LSA (white small colony).

Table 1: Summary of the type and total amount of samples collected from the study areas.

S.No	Sample type	Sample collection site	Total sample
1	Pooled milk	Dairy farm	80
2	Cheese	Market	50
3	Yogurt	Shop	50
	Total		180

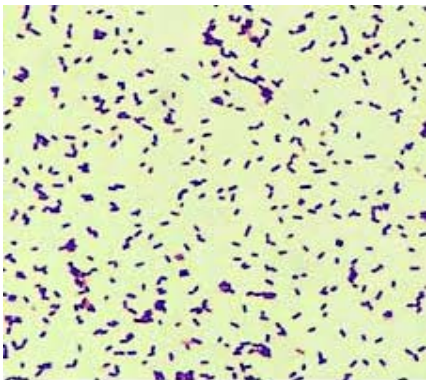

Figure 4: Rod shaped colonies on grams reaction slide show.

rod and positive for the grams stain (Figure 4) [29].

MALDI-TOF Identification

From twenty-three suspected colonies, all isolate were negative for *Listeria* by MALDI-TOF identification system although some were positive for another bacteria called *staphylococcus, Enterobacter, bacillus, enterococcus* which have similar characteristics with *Listeria-on-Listeria* selective agar and morphologically by conventional biochemical tests as indicated on the table of the MALDI-TOF result on annex 3 and 4.

Questioner Survey Result

As the result indicated the general farming system and the incidence of exposure to *Listeria* was examined by animal breed 22.5% were local and 77.5% were crossed, lactating length 66.35% of animal have an average of one year lactating length and 28% were two year in lactating, age of the animal 22.5% of the animal are below 5 year of age and 25.5% of milking cow are aged 5, milk yield 22.5% of milking cow gives 4-10L milk per day and 58.8% gives above 15L of milk per day, washing practice before milking 100% of the farm we visited have a good practice on cleaning the area, teat of milking cow and cleaning milking materials, disease syndrome seen which are associated with *Listeria* 18.8% of the farms face a problem related to abortion,17.5% of calf death reported on the farms and 42.5% of cow were diseased and milk delivery method in use shows 37.5% milk delivered by sales personnel, 32.5% of the customer comes to buy and the rest 30% use selling mechanism by contract with companies (Figure 2).

Discussion

In the present study, from the total of 180 animals pooled milk and milk product examined, 23(6.0%) were presumptively positive for *Listeria* by the conventional biochemical test isolation methods nonetheless all were negative by Bio log identification system. Thus, our result shows 0% *Listeria* in the studied target populations.

Beside the isolation process follows OIE and ISO standard method of isolation and identification of *Listeria* by the method of using pre-enrichment and enrichment broths advances made in the selective isolation of *Listeria* from samples from the food chain, there is still room for improvement in a number of areas. No single procedure can be credited with being sensitive enough to detect *Listeria* from all types of food [30].

The analysis of chess sample showed 1.6% was contaminated with only *L. innoca*. The low contamination rate might be attributed to the fact that death of most of the organism occurred in cheese tend to have lower PH and it is also related with portal heat treatment. Our finding is in agreement with previous reports that indicated a prevalence rate of 0-10% depending up on the type of cheese and production process [31].

Also, in previous studies on raw milk there have been low prevalence with compared to other animal origin food items like beef meat, pork meat and fish [32]. The lowest prevalence of *Listeria species* in cream (23%) and cheese and raw milk (0.0%) was reported in Addis Ababa [33]. and Gondar town [34]. The highest and the lowest contamination of *Listeria species* was reported from Gondar town with highest prevalence 25% [35]. And the lowest prevalence 0.0% [34]. which may be due to study period. The highest prevalence of *Listeria species* in beef meat was reported in Addis Ababa [33] with prevalence of 62%, but the lowest prevalence was reported in Gondar town [35] with a rate of 25%. Higher prevalence of *Listeria species* in cream (43%) and in egg (32%) was reported in Amhara region [34] and Addis Ababa [36], respectively.

As indicated above with the agreement of the majority of the studies milk and milk products show less prevalence than other animal origin food items for human consumption. Beside the fact that our samples were smaller in number than the other studies done due to limited amount of resources available in the laboratory, short study period compare to is the culturing process of *Listeria* requires selective pre enrichment procedures which takes around more than one week for single sample. Some of the disadvantages of this group of methods include the relatively long period of time that the protocols require for completion, several 'hands-on' manipulations, the requirement for many different chemicals, reagents and media, the possibility of contaminating microorganisms in the sample masking the presence of the target ones, including overgrowth, the potential overlook of atypical variants of the target organism and the relative subjectivity involved when interpreting typicality of colony on selective and differential agar plates [30].

Also in the majority of the studies, *Listeria* was detected using traditional culturing methods, (none of the studies ever use biology method by MALDI-TOF). An alternative method for the rapid identification of *Listeria species* is the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), which is increasingly being used worldwide in microbiology laboratories. MALDI-TOF MS identification systems are based on the comparison of the tested isolate mass spectrum for proteins, and also for lipids, with reference databases. For *Listeria* isolates, the genus and species could be accurately and rapidly identified with a validation of one MALDI-TOF MS for *Listeria* system by the WHO8

Table 2: Results on questioner about risk factors of *listeria* on different farms.

Parameter	Practice	Frequency	Percent	Cumulative	χ2	P-value
Breed type	Local	18	22.5	22.5	8.84	0.03
	Crossed	62	77.5	100		
Lactating length	One year	53	66.3	66.3	1.18	0.757
	Two year Two year	23	28.7	95.0		
	Above two years		3	4.8	100	
Age milking cow	<5 year	18	22.5	22.5	1.44,	0.47
	=5 year	20	25.0	47.5		
	>5 year	42	52.5	100.0		
Wash before milking	Yes	80	100	100	Yes	80
Milk yield	4-10 liters/day	18	22.5	22.5	1.47	0.47
	11-15liters/day	15	18.8	41.3		
	>15 liters/day	47	58.8	100		
Milking type	Manual	63	78.8	78.8	0.55	0.44
	Automated	17	21.3	100		
Syndrome associated with listeria	Circling	0	0	0		
	Decrease milk	17	21.3	21.3		
	Abortion	15	18.8	40.0		
	Death of calf	14	17.5	57.5		
	Diseased cow	34	42.5	100.0		
Delivery method	Each customer out	26	32.5	32.5		
	By sales person	30	37.5	70.0		
	Contract with companies	24	30.0	100.0		

Collaborating Centre [24].

Very recent and none of the studies in my site have been done using this automated system. Conventional tests which are in general considered less sensitive methods. A very limited number of studies used other confirmatory test for isolation and identification of *Listeria*. Moreover, variation in the sensitivity of culture detection methods can influence the prevalence and consequently the observed heterogeneity [37]. Even if, absent of *Listeria* in this study may not fully indicate the absence of *Listeria* in the study area, we dare to question the reliability of *Listeria* prevalence findings in the country where solely done with biochemical tests.

Conclusion and Recommendations

Listeria is the emerging pathogenic bacteria in human as well as in animals. Although *Listeria* commonly found in food of cattle origin like milk and meat samples on previously done researches, it could potentially be contaminated and affect peoples and other animals. This study finds presumptively positive findings upon biochemical tests but reacted negatively when the presumptively positive samples subjected to the MALDI-TOF identification system as a confirmatory test. The MALDI-TOF found those presumptive organisms as staphylococcus, *enterococcu, Bacillus* and *Candida*. Our finding pop up many questions regarding the reliability of some of the bacteriological tests unless incorporated with other tests and to see an in-depth of the associated risk factors. Therefore, based on the above conclusion the following recommendations are forwarded:

- Isolation and identification of *Listeria* organism should not be reached to the conclusion relying only on biochemical test as there are other bacteria characteristically similar with *Listeria*.
- The milk hygienic condition must be improved, as it appears to be easy for organism transmission.
- Even if my result showed 0% prevalence, I highly recommend the dairy farms, cheese on local market and organization of milk pasteurization to be vigilant about emerging pathogens and further research should be done.

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