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Detection and Isolation of *Listeria monocytogenes* from bulk tank milk samples of dairy farms.

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Abstract

The present study was performed on Forty three bulk tank milk samples collected from different private dairy farms. The samples were cultivated on the selective media, broth and agar plates of *Listeria* species. *Listeria* species could be isolated from the examined samples (17) isolates; these were represented by a percentage of (39.5%). *Listeria* species included (*L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, and *L. murrayi*). While the detection and isolation of *Listeria monocytogenes* (6) isolates, that represented (13.95%). The identification of *L. monocytogenes*was based on the use of the morphological, biochemical, pathological and serological characters.

Keywords: detection, isolation, identification, *Listeria monocytogenes*, bulk tank milk samples.

Introduction

Hitchins, 1998 and Seeliger and Jones, 1986 were determined that, The genus *Listeria* includes seven different species (*L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, and *L. murrayi*). Both *L. ivanovii* and *L. monocytogenes* are pathogenic in mice, but only *L. monocytogenes* is consistently associated with human illness

L. monocytogenes is a ubiquitous bacterium (Larpent, 1995). *L. monocytogenes* may be found in the intestines of animals and humans without causing illness (Bertolussi, et al., 1985). It is widely distributed in many natural and man-made environments such as soil, water, sewage, vegetation and silage where it can survive for long periods of time (Bertolussi, et al., 1985) and (Dattaand Benjamin, 1997). *L. monocytogenes*is a facultative intracellular gram-positive bacterium that invades, replicates, and multiplies in a variety of mammalian cells (McLauchlin 1997). *L. monocytogenes* has again become a major public health concern, after a series of high profile outbreaks. In 1980 some steps were taken to reduce contamination of food, and it appeared that this pathogen was under control. But a spate of recent outbreaks and recalls has demonstrated that this bacterium still poses a significant health risk (Mead, et al., 1999). This is partly due to its widespread occurrence in nature and its ability to grow at refrigeration temperatures (Farber and Pertenkins, 1991). *L. monocytogenes* is a food-borne pathogen. The virulent nature of listeriosishas a mortality rate of up to 33% (CassidayandBrackett, 1989) and (Liewen and Plautz, 1988). Thus the detection and isolation of *L. monocytogenes* has a very important value in the restriction of spreading it (Anthony Hitchins and Karen Jinneman, 2011).

The aim of this work is the rapid detection and the prevention of the spreading of Listeriosis.

Material and Methods

Samples:

Forty three bulk tank milk samples were collected from different private dairy farms in screw capped bottles under aseptic condition.
All collected samples were kept in an insulated container with ice packs and transferred to the laboratory without delay to be subjected to the bacterial culturing.

Bacteriological Culture:

Samples treatment: Samples are refrigerated at 4 °C for handling, storing, and shipping materials to be analyzed for *L. monocytogenes*, which will grow, although slowly, at this temperature if other conditions permit (Gray et al., 1948). However, if the samples are already frozen, it should not be thawed until analysis. *L. monocytogenes* has an optimum growth temperature of 30 – 37 °C in a neutral or slightly alkaline pH (i.e. pH ≥ 7). However, *L. monocytogenes*can also grow at refrigerated temperatures < 5 °C. The generation time (i.e. growth rate to double population) can vary from 1.1 to 131 hours depending on temperature and other factors (Lou and Yousef, 1999) and (International Commission, 1996). The treated milk samples were streaked on sheep blood agar plates and incubated at 37 °C for 24 to 48hr. The growing colonies were sub-cultured on Listeria Oxford media base (Oxide). The inoculated plates were incubated at 37 °C for 24 to 48 hr. The growing colonies on Oxford agar media plates were suspected as *L. monocytogenes* or other *Listeria* species. The suspected colonies were suspended in the specific Listeria Enrichment Broth (Difco). The inoculated broths were incubated at 30 °C for 1week with daily observation (Twedt, R. M., A. D. Hitchins, and G. A. Prentice., 1994) and (AOAC Official Method 993. 12., 2000). The purity of isolated strain of L. *monocytogenes* was obtained by the re-cultivation on the Oxford agar media plates, from the turbid test tube broths after the incubation period (Walker, 1999). The obtained pure colonies were identified according to the morphological, the biochemical, the pathological and serological characters.

The morphological Characters of L. monocytogenes on Oxford agar:

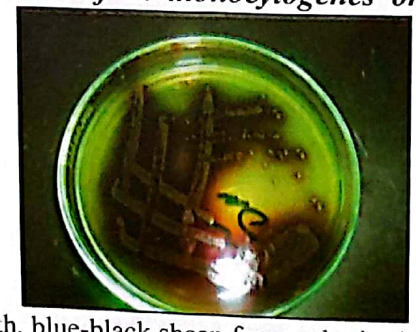

Fig (1):Characteristic smooth, blue-black sheen from colonies by obliquely transmitted light

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Motility test

The micro-organism was cultured onto semi-solid media to observe any motility as discussed by (Quinn et al., 1994). Hanging-drop method was also applied to confirm the type of mortality adopting (Quinn et al., 1994). While in case of *L. monocytogenes*was performed on test tube broths at 20-25 °C, tumbling motility of *Listeria* cells was determined as described by (Rowan and Anderson, 1998), Fig (2).

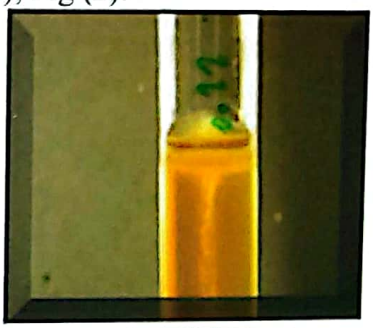

Fig (2)

Microscopic examination:

Gram's stained films from the colonies showed Gram-positive, Non-spore forming short rods microorganism, Fig (3).

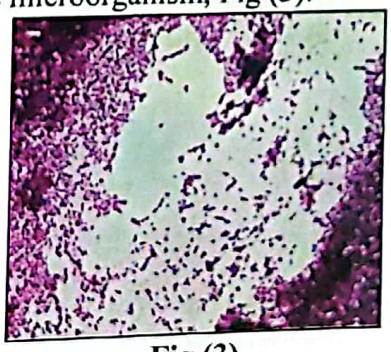

Fig (3)

Blood heamlysis:

The colonies of*L. monocytogenes*are Beta hemolytic in nature. Which is weak on sheep and horse blood agar (Dulce et al., 2008).

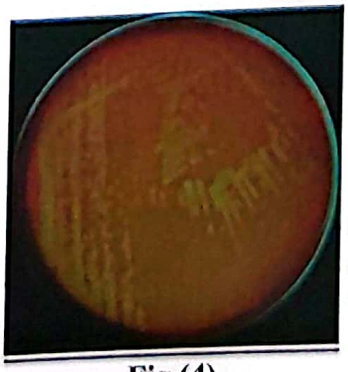

Fig (4)

The identification of L. monocytogens was carried out according to the following:

Biochemical Characters:

Sugar fermentation test:

L. monocytogenes ferment Esculine, in the Oxford agar plate resulting in blue-black sheen, Fig (1).

L. monocytogenes ferment Rhamnose, but not ferment Xylose and Mannitol, Table (2).

Catalase test:

Catalase test was applied to differentiate *L. monocytogenes* from micrococcus species of bacteria. A small amount of a bacterial colony (18 to 24 hours old) on a clean glass slide, one to two drops of 3 % hydrogen peroxide was added

Catalase production was determined by observing the occurrence of O_2 bubbles, as described by (Bubert et al. 1997).

The oxidase test:

One drop of oxidase reagent was added to 18 to 24 hours colony of bacteria Blue to dark purple color is considered as positive result.

The CAMP test (CAMP:)

The test was performed using standard procedures by streaking out bacteria perpendicular to *Staphylococcus aureus* on 5% sheep blood agar plates, incubated at 37°C for 24 hr. and observing zones of augmented haemolysis, as described by (Bubert et al., 1997), Fig (5).

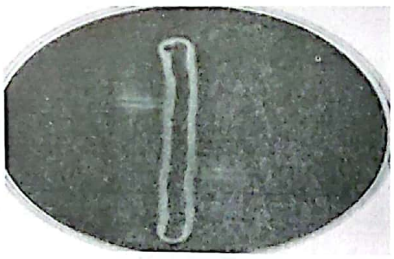

Fig. (5)

Pathological Characters:

Mouse pathogenicity test:-

Pathogenicity test for *L.monocytogenes* Using Swiss white mice. Pathogenicity was determined by a modification of the method of **(Ralovich1984).** Isolates were grown for 24 hr. at 35°C in Trypticase soy broth (BBL Microbiology Systems: Bacterial Broth of *L.monocytogenes*) plus 0.6% yeast extract. Cultures were concentrated 10-fold by centrifugation and suspended in 0.1% peptone.

Five Swiss white mice were each given 0.1 ml of the suspension, containing 109 cells, intraperitoneally (i.p.). The mice were observed for 1week, and deaths were recorded. Strains that killed three or more mice were considered to be pathogenic. In each experiment, control mice were injected with known pathogenic and nonpathogenic strains; experiments in which either set of controls gave inappropriate results were disregarded (Conner et al., 1989).

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***Listeria* Rapid Test (Oxoid, 2002):**

a-Primary Enrichment:

Twenty-five ml of milk samples were homogenized in a sterile wide mouth jar with 225ml of Fraser Broth (FB-CM895) supplemented with Half Fraser Selective Agent (SR 166A). The mixture was incubated at 30 °C for 21 hours.

b- Secondary Enrichment:

0.1ml of FB culture was inoculated into 10 ml volume of Buffered Listeria Enrichment Broth (BLEB-CM897) supplemented with BLSB Selective Agent (SR141E). The incubation was done at 30 °C for 21 hours.

C -Antigen Extraction:

Two ml of upper region of BLEB culture were transferred to a small test tube incubated in a water bath adjusted at 80 °C for 20 minutes, then tempered at room temperature.

d-*Listeria* Device:

The Device was left at room temperature shortly before use. 135 µl of BLEB extract were transferred (micropipette) onto a pad in the Sample Window. The antigen/latex complex was moved by capillary action to both result window and control window. After 20 minutes the device was examined for appearance of a blue line in the control window and result window.

Appearance of a blue line in the control window indicated the device has worked correctly. Appearance of blue line in the result window indicated *Listeria* positive sample.

How does Listeria Rapid Test work?

1. Primary (Fraser Broth) and secondary (Buffered *Listeria* Selective Broth/BLSB) enrichment stimulate the productivity of B-flagella antigen.
2. Heating cultures of BLSB at 80°C for 20 minutes facilitate the extraction of B-flagella antigen (Seeliger and Jones, 1986; Holbrook et al., 1993; Parry et al., 1993 and Holbrook et al., 1994).
3. *Listeria* except *L. grayi* possesses a common B-flagella antigen which is not shared by other bacteria.
4. The specific monoclonal Listerial antibodies in the device (coated latex) have been tested against B-flagella antigen giving a blue line in a result window in case of positive result.

Fig. (6)

Superiority or necessity to Listeria Rapid Test:

It is simple system, unlike EIISA no reagents, mixing or washing steps are required.It is a sensitive test which can be detect low level of Listerial cells (two cells/ml or gram).It gives a clear visual rapid result, the time consumed (43 hours) less than one third the time taken by traditional methods (about one week).

Judgment of Listeria Rapid Test:

It is designed for the detection of *Listerias* in foods including milk, dairy products, meats, poultry, vegetables and fishes. Besides environmental samples (stainless steel, plastic, rubber, glasses, wood, air filter). It is recommended by AOAC Research Institute (USA).

Results

Isolation of*Listeria* species from bulk tank milk samples, 17 isolates that represented in a rate of (39.5%). **As shown in Table 1**

The recorded results were obtained on the following percentages **(Tables 1 and 2)**: *L. monocytogenes*(13.95%), *L. ivanovii*(7%), *L. innocua*(4.65%), *L. welshimeri*(2.33%), *L. seeligeri* (4.65%).

Table 1 Isolation rates of *Listeria* species

Species	No. of Isolates	The percentages
L. monocytogenes	6	13.95%
L. ivanovii	3	7%
L. innocua	3	7%
L. welshimeri	2	4.65%
L. seeligeri	1	2.33%
L. grayi	2	4.65%
L. murrayi	0	0

Thedifferentiation of *Listeria species* according to fermentation of sugar, blood haemolysis and virulence test in mice, Table 2.

*L.monocytogenes*fermentRhamnose, Beta haemlytic and viulent to mice, while *L.innocua*can'tfermentRhamnose,Table 2.

Table 2 Differentiation of *Listeria* species

Species	Xyl.1	Rha.2	Man.3	β-hem.4	Vir.5
L.monocytogenes	-	+	-	+	+
L.innocua	-	V	-	-	-
L.seeligeri	+	-	-	W	-
L.welshimeri	+	V	-	-	-
L.ivanovii	+	-	-	+	-
L.grayi	-	-	+	-	-
L.murrayi	-	V	+	-	

V = variable reaction. W =weak

1-Xylose 2- Rhamnose 3-Mannitol 4-β-hemolysis 5-Virulence

Discussion

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The present study was designed for detection and isolation of*L. monocytogens* in bulk tank milk samples, out of 43 bulk tank milk samples collected from different private dairy farms, 17 isolates of *Listeria* species with an incidence of (39.5%) was recorded in (Table 1). This result showed a higher incidence of *listeria* species, than recorded by (Mahmood et al., 2003) Tabulated a rate of (37.5%). The great variationin the incidence of isolation of *listeria* species may be due to the contamination of the samples with the microorganism from different sources or improper cleaning and disinfection of the udder during samples collection…etc. Therefore, the higher incidence of *Listeria* species could be attributed to contamination caused by improper cleaning cloth, other working surfaces and more human contact (Lowry and Tiong, 1985).

Listeria species can be a common contaminant in the dairy environment, both on the farm and in the processing plant. On the farm, the animals themselves can be the source of infection as the *Listeria* species are usually found in manure and improperly fermented silage. In the dairy plant, Listeria is most frequently found in moist environments or areas with condensed or standing water or milk, including drains, floors, coolers, conveyors and washing areas. But also the *Listeria*specieswas found in foods which have received minimal or no processing and could be considered as a source for cross contamination occurring at the food chain (Schuchatet al., 1992).

The differentiation of *Listeria* species by the morphological, the biochemical and the pathogenicity was carried out according to (Dattaet al., 1987), (Dattaet al., 1991), (Peterkin, 1991) and (Merker, 1998); the recorded results were obtained on the following percentages (Tables 1 and 2): *L. monocytogenes*(13.95%), *L. ivanovii*(7%), *L. innocua*(4.65%), *L. welshimeri*(2.33%), *L. seeligeri* (4.65%).

The reported rate of *L. monocytogenes* was (13.95%), which was higher than that reported by (Schultz, 1967); examined 1,004 raw-milk samples from dairy cows and detected

L. monocytogenes in only 10 samples. While *L. monocytogenes*was isolated from raw milk in the rate of 12% by (Peggyset al., 1986).

*L. monocytogenes*is a food-borne pathogen which presents a serious threat to consumer (health and safety) and has been implicated in several deadly out-breaks in the United States and around the world.

The ability of *L. monocytogenes* to multiply at refrigeration temperatures could be considered of a significance in food intended for consumption without further cooking and foods which have received cooking presumed sufficient to eliminate listeria, but nevertheless intended be received further cooking prior to consumption where the potential competitive micro flora has been largely eliminated and thus even low numbers could pose a potential hazard if proper storage conditions are not adhered to (Schuchatet al., 1992).

Pathogenesis of L.monocytogenes:-

L. monocytogenes is a highly invasive intracellular pathogen. The bacterium then polymerizes actin filaments at one end to form long actin tails that propel it through the cytoplasm (Salyers and Whitt, 2002). The adherence and invasion are

mediated by membrane proteins "calinternalins": InlA and InlB. The forced phagocytosis brings *L.monocytogenes* into the host cells encased in a vacuole. The bacteria then escape the vesicle by a protein hemolysin, listeriolysin O (LLO), (Salyers and Whitt, 2002) and (Kuhn and Goebel, 1999). LLO is a pore forming cytotoxin. It is also responsible for the zone of beta-hemolysis seen on blood agar plates. The gene encoding LLO is named "hly" for "hemolysin." (Kuhn and Goebel, 1999).

An early study suggested that *L. monocytogenes* is unique among Gram-positive bacteria in that it might possess lipopolysaccharide (Wexler and Oppenheim, 1979), which serves as an endotoxin. Later it was found to not be a true endotoxin. *Listeria* cell walls consistently contain lipoteichoic acids, in which a glycolipid moiety, such as a galactosyl-glucosyl-diglyceride, is covalently linked to the terminal phosphomonoester of the teichoic acid. This lipid region anchors the polymer chain to the cytoplasmic membrane. These lipoteichoic acids resemble the lipopolysaccharides of Gram-negative bacteria in both structure and function, being the only amphipathic polymers at the cell surface (Fiedler, 1988) and (FarberandPertenkins, 1991).

In humans, the infection with *L. monocytogenes*causing illness known as (listeriosis). The severity of listeriosis ranged from a mild flu like sickness (sometimes leading to a carrier state) to severe manifestations. The severe forms of human listeriosis present as meningo-encephalitis followed by septic infections and occasionally isolated organ involvement (Mahmood et al., 2003). Groups at higher risk are pregnant women, neonates, adults with underlying diseases like cancer, AIDS, diabetes, chronic hepatic disorder, transplant recipients, old age, and other immune-compromised individuals. Death is rare in healthy adults but can occur at as high as 30% in persons at highest risk (Demetrioset al., 1996). In order to minimize human listeriosis, foods should be cooked to an internal temperature of 70 °C for more than 20 minutes to ensure destruction of *L. monocytogenes*.

Influenza-like symptoms, including persistent fever, usually precede the onset of the aforementioned disorders. Gastrointestinal symptoms, such as nausea, vomiting, and diarrhea, may precede more serious forms of listeriosis or may be the only symptoms expressed. Gastrointestinal symptoms were epidemiologically associated with use of antacids or cimetidine. The onset time to serious forms of listeriosis is unknown, but may range from a few days to three weeks. The onset time to gastrointestinal symptoms is unknown but probably exceeds 12 hours. The infective dose of *L. monocytogenes*varies with the strain and with the susceptibility of the victim. From cases contracted through raw or supposedly pasteurized milk, one may safely assume that, in susceptible persons, fewer than 1,000 total organisms may cause the disease. *L. monocytogenes* may invade the gastrointestinal epithelium and once the bacterium enters the host's monocytes, macrophages, or polymorpho-nuclear leukocytes, it becomes blood-borne (septicemic) and can grow.

Its presence intracellularly in phagocytic cells also permits access to the brain and probably transplacental migration to the fetus in pregnant women. The pathogenesis of *L. monocytogenes* centers on its ability to survive and multiply in phagocytic host cells. It seems that *Listeria* originally evolved to invade membranes of the intestines, as an intracellular infection, and developed a chemical mechanism to do so.

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Listeriae are responsible for approximately 27.6% of the total deaths attributed to food-borne illness. In comparison, food-borne salmonellosis has a fatality rate of only 0.04%. Even though salmonellae account for more than 500 times as many cases of food-borne illness as *L. monocytogenes*, they cause only about 54 more deaths per year (Mead et al., 1999). When listeric meningitis occurs, the overall mortality may reach 70%, from septicemia 50%, and from prenatal / neonatal infections greater than 80%. In infections during pregnancy, the mother usually survives.

Conclusion and recommendations:

*Listeria monocytogenes*is an intracellular opportunistic organism found as contaminants of environment and different materials. Correct practices with respect to silage production and milking are essential for preventing introduction of *Listeria* into the herd, its spread within the herd, and its entry into milk. *Listeria monocytogenes*can multiply at a higher rate in poorly stored silage. It can survive at a refrigerator temperature. These contribute as a source of infection to animals so that listerial infection is most prevalent during winter (cold season). Generally, the clinical forms of listeriosis include septicemia of neonates, neonatal death, abortion, septicemia and diarrhea of ewes and neurological diseases (circling disease).

Based on the above conclusions, the following recommendations are forwarded; As *Listeria monocytogenes*does not multiply to any significant extent in effectively preserved silage, which is characterized by anaerobic storage, high concentration of organic acids and a pH below 4.5, the silage should be stored under these characterized conditions to maintain its quality. Incorporation of silage into the diet should be gradual and provision of green pasture should be encouraged. Susceptible animals should not be exposed to wet, cool and unhygienic environment. Silage that is obviously decayed should be avoided from the environment. Also it is recommended that farmers wash teat of cows, hands and other milking equipment adequately with a suitable antiseptic to prevent contamination of raw milk. Finally the infected animal must be isolated as it is asource of infection. Based on the study findings: improving quality of raw milk, programs should be conducted. As the milk may be responsible for transmission of some health hazard microorganisms that increase the need for highly effective diagnostic procedures to maintain the health of milk consumers. Reliable and accurate isolation and detection techniques are important in the surveillance of *Listeria monocytogenes*andlisteriosis. Standard and hygienicoperating methods in the farming, processing andmarketing of foods are the way forward to reduce the incidence of listeriosis.

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كشف و عزل الليستيريا منوسيتوجين من عينات لبن من تانكات المزارع الحلابة

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قد أجريت هذه الدراسة على ثلاثة وأربعون من عينات لبن من تانكات المزارع الحلابة التي تم جمعها من مختلف مزارع الألبان. تم الانتهاء من زراعة العينات. أنواع الليستيريا شملت (*L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, and *L. murrayi*).