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College of Veterinary
Medicine

Detection of *listeria monocytogenes* in Raw milk and Dairy products in Kerbala Province

Thesis

Submitted to the council of the College of Veterinary Medicine at University of Kerbala as a Partial fulfillment of the Requirement for the Degree of Master in the Sciences of Veterinary Medicine in Veterinary Public Health

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بسم ٱللَّه الرَّحْمٰنِ الرَّحِيمِ

﴿ وَقُلْ رَبِّ زِدْنِي عِلْمًا ﴾

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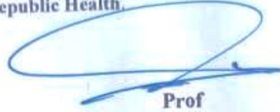

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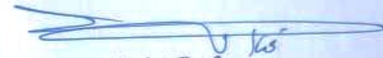

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I hereby declare that this thesis is my origin work except for equations and citations which have been fully acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at University of Kerbala or other institutions.

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Dedication

To the one who conveyed the message and fulfilled the trust, and advised the nation, To the prophet of mercy and the light of the worlds, "Our Master Muhammad, may God bless him and grant him peace." ….

To the souls of the martyrs of the security forces and Al hashed AL_shaaby, whose blood perfumed the homeland, had it not been for their sacrifice, we would not all enjoy safety and we would not have been able to complete our educational path….

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List of Abbreviations

Abbreviation	Complete words of terms
PH	hydrogen ion concentration
DNA	Deoxyribonucleic acid
Spp	Species
Oxoid	Oxford agar
LLO	Listeriolysin
PCR	Polymerase chain reaction
ELISA	Enzyme linked immune sorbent assay
ETC	Electron transport chain
Mg/kg	Milligram per kilogram
M RNA	Messenger RNA
L.M	Listeria monocytogenes
Act A	Actin
WST	White side test
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
µl	Microliter
ml	milliliter
GD	Genomic DNA
^{o}C	Celsius degrees
IFN-g	Interferon gamma

LD	Lethal dose

Abstract

The objective of the present study was to detection of *Listeria monocytogenes*. in raw cows' milk and dairy products.

Two hundred fifty Samples of raw milk and dairy products which include (yogurt, cheese, Butter) were collected randomly in interval from 1st January to 30 June 2022.

In Kerbala province the samples of milk were collected from cows in local farms while the samples of dairy products were collected from markets and sell points, All samples were treated according to standard protocols of *Listeria monocytogenes* and delivered to laboratory for analyzing.

The study of physical features for the samples of milk and dairy products which contaminated with *Listeria Monocytogenes* were revealed change in color, smell and taste, while the biochemical reactions have been showed decrease in pH, and increase in chloride while, catalase and white side test given positive reaction.

The bacteriological study which included isolation and identification of *listeria monocytogenes* from animal sources by using selective media for *listeria monocytogenes*, which is the (oxford agar) from (Hi media) Company for culturing. The isolates of *Listeria Monocytogenes* was showed on selective media as black colonies also all isolates of *Listeria Monocytogenes* were given positive results for catalase test.

Molecular study of *Listeria Monocytogenes* was done by conventional PCR technique after DNA extraction by using a DNA extraction Kit.

Four specific duplex primers (Hly-1-f, Hly-2-R, List-F, List-R) were designed on the basis of a divergent region of the *L.*

monocytogenes genome that was by means of microarray analysis directed to the Hly gene, isolates of *L. Monocytogenes* were identified and study of genetic variation between local *listeria monocytogenes* isolates which was carried out using the DNA sequencing process which confirming contamination of raw milk and dairy products samples.

The sensitivity test of all bacteria of isolates to antibiotics was studied, as it was observed that they were sensitive to some antibiotics include [Neomycin with sensitivity zone (28 mm), Ciprofloxacin (10 mm), Gentamycin (15 mm), Chloramphenicol (20 mm)] However, resistance to the antibiotics [Amoxilin with Resistance zone (10 mm), Ceftriaxone (5mm)].

The incidence of *listeria monocytogenes* varied by area from 32% in al-Gadwal Algarbi to 20 % in each of Al-Husainia, Al-Hindia and Ain Altamer with total incidence of 10 %.

The study was concluded contamination of raw milk with *Listeria monocytogenes* in Karbala city and its outskirts, milk and dairy products are one of the main sources for *L. Monocytogenes* spreads to human. the highest percentage of *listeria Monocytogenes* occurrence was in winter season more than in the summer, especially January and February.

Chapter one: Introduction

Introduction

Listeria monocytogenes is a zoonotic facultative anaerobic intracellular. non-spore forming foodborne pathogen causing serious illness worldwide (Baer. *et al*.,2013). *Listeria monocytogenes* is a small, coccoid to rod-shaped, gram-positive organism exhibiting a tumbling motility at room temperature. The organism is often designated as a coryneform contaminant because of its unique morphology (Pirie,1940). Identification of new isolates may be achieved by examination of cellular morphology, colonial morphology and hemolytic reaction on sheep or horse blood agar and blue-green coloration of colonies when viewed microscopically with oblique lighting on tryptose agar (Seeliger *et al*., 1986). The organism is catalase positive, oxidase negative, and the fermentative metabolism of glucose results in the production of mainly lactic acid, but no gas is produced from a number of other sugars (Griffiths, 1989).

The genus *Listeria* contains 17 species, but *Listeria monocytogenes* (*L.M*) is the only known pathogenic strain (Townsend,2021). These strain include: *Listeria monocytogenes, Listeria ivanovii, Listeria seeligeri, Listeria innocua, Listeria welshimeri, Listeria grayi, Listeria marthii, Listeria rocourtiae, Listeria fleischmannii, and Listeria weihenstephanensis* (Halter, 2013; Zhang *et al*., 2007).

Many researches have been recorded that *L. monocytogenes* causes large outbreaks of Listeriosis with a mortality rate of 9% to 44% (Clark *et al*.,2010). The most susceptible domestic species are sheep, goats and cattle. Listeriosis manifests itself clinically in ruminants as encephalitis, neo-natal mortality (abortion) and septicemia, the most common clinical form in cattle is encephalitis (Hunt *et al.,*2012). Most women present with a bacteremic illness consisting of fever, chills, headache and leukocytosis with a 6–7-day illness before diagnosis (McLauchlin, 1990).

Multiple outbreaks of listerial gastroenteritis have been described and typically occur in healthy persons (Lorber, 1990). Following an incubation period of 6–49 h most patients present with diarrhea, fever, abdominal pain, chills, headache and myalgia’s, most cases of listerial meningitis/meningoencephalitis are seen in patients >50 years of age and fever, altered sensorium and headache are the predominant symptoms (Brouwer *et al.*, 2006).

L. monocytogenes is psychotropic and can grow in acid conditions (George *et al*., 1988). The behavior of *L. monocytogenes* is related to its initial concentration and the pre-incubation conditions (Gay *et al*.,1996). *L.Monocytogenes* have ability to survive and grow at refrigeration temperatures and tolerance to the preserving agents, sodium chloride and sodium nitrite makes *Listeria* of particular concern as a post contamination agent for refrigerated food and ability of organism to grow at refrigeration temperature is not well documented (Talat, 1990). Since food borne listeriosis was first documented in 1981 (Dalzini *et al*., 2017). Numerous food borne outbreaks of *L. monocytogenes* have been documented worldwide (Denny *et al*., 2008). Meat, poultry, dairy, and vegetable products have all been implicated as vehicles of listeriosis (Gianfranceschi *et al*., 2006).

Raw milk and milk products especially cheese made from unpasteurized milk of cow and sheep are widely considered as an important sources of *L. monocytogenes* contamination and a vehicle of listeriosis (Pinto *et al*., 2009). The presence of *L. monocytogenes* in raw milk and pasteurized cheeses may be related to numerous parameters such as contaminated raw milk, defective pasteurization, and post processing contamination (Dalzini *et al*., 2017). The pathogenicity of *L. monocytogenes* is determined by several virulence factors, such as: listeriolysin O (LLO), internalins, phospholipases, actin assembly inducing protein (ActA), invasion-associated protein (p60) and regulatory system for gene expression of virulence (PrfA) (Liu ,2006).

The prevalence of pathogens in milk is influenced by numerous factors such as farm size, number of animals on the farm, hygienic conditions, farm management practices variation in sampling and types of samples evaluated, differences in detection methodologies used, geographical location, and season. However, in spite of the variation, all of these surveys clearly demonstrated that milk can be a major source of foodborne pathogens of human health significance (Oliver *et al*., 2005). One specific characteristic of *L. monocytogenes* that appears to be critical to its ability to cause human foodborne illness is its capacity to grow at low temperatures, *L. monocytogenes* has been shown to grow at temperatures ranging from −0.4 to 45◦C (Gray *et al.*, 1966). Although many different conventional testing methods have been developed for the detection and enumeration of *L. monocytogenes* from food, these have relied almost exclusively on the use of specific culture media followed by a series of tests for confirmation. Conventional plate counting

methods are laborious, time consuming and sometimes underestimate the numbers (Choi ,2003).

Listeria Monocytogenes considered a psychrotolerant organism as its optimum growth temperature is in the range of 30 to 37◦C, while it has the ability to grow at temperatures <15◦C (Junttila *et al.*, 1988). Milk and dairy products are excellent sources of essential nutrients and casein, a major milk protein, because of their high nutritional value, they are very suitable for development of microorganisms, including pathogenic bacteria as Listeria species (Kasalica *et al*.,2011). Resulting in listeriosis in both human and animals (Ryser, 2007). *L.Monocytogenes* has been involved in many outbreaks and sporadic cases of diseases primarily associated with the consumption of pasteurized milk, cheeses made from unpasteurized milk and other dairy based products that serve as good medium for the growth and survival of many pathogenic organisms in both industrialized and developing countries (Makino *et al*., 2005).

The antibiotic resistance of the pathogen is a significant public health concern, the first antibiotic resistant *L. monocytogenes* strain was reported in 1988. Since then, an increasing number of resistant strains isolated from foods, animals and humans. The studies in the last decade have provided sufficient evidence to document that Listeria spp. including *L. monocytogenes* are resistant to various antibiotics such as rifampin, cephalothin, nalidixic acid, penicillin G, sulphamethoxole/trimethoprim, chloramphenicol, tetracycline, oxacillin, lincomycin, flumequine, clindamycin, cefotaxime, cephalosporine, ampicillin, erythromycin, gentamycin, methicillin, teicoplanin, tetracycline and vancomycin (chen *et al*.,2017).

Aims of Study:

In Iraq raw milk is an important vehicle for transmission of various diseases due to insufficient hygienic standards in dairy farms. Therefore, the present study was conducted to.

1. Determine the occurrence of *L. monocytogenes* in raw milk, dairy products in farms and livestock fields, selling points and markets.

2. Molecular detection of isolated *Listeria monocytogenes* from raw milk and milk products.

3. Assess the genetic relationship between *L. monocytogenes* isolates.

4. Evaluate the antimicrobial resistance of potentially virulent *L. Monocytogenes* isolates.

Chapter two: Review of the Related Literature

2. Review of the Related Literature

2.1 History of *listeria monocytogenes*

The first description of the organism was conducted by Murray in 1926 which called then bacterium monocytogenes, it has been diagnosed as *listeria monocytogenes* due to increasing the number of monocytes cells, then it is included in corynbacterium family, then it was added to the Gram-positive Bacillus group Listeriaceae (Iqbal,2005). The specific role of this bacterium as a dangerous pathogen was discovered in 1949 when an infection of listeriosis occurred in newborns in Germany at the University of Halle where a foreign body was observed at the Institute of Pathology (Reiss *et al*.,1951).

There are four major area of concern for this bacterium (Algebori,2005):

- In microbiology: Listeria Monocytogenes cause serious disease of human and animals which is hard in treatment.

- In food microbiology: Listeria Monocytogenes food borne bacteria because it’s presents in many food materials.

- In cell biology: listeria is facultative bacteria and intracellular organism which have cross junctions and interactions within host cells.

- In immunity: mediated immunity had been conducted from cells through listeriosis model (Hof, 2003).

2.2 Taxonomy of listeria genus

For a long time after the listeria genus was discovered, it only contained listeria monocytogenes species because of this species' ability to reduce nitrate. However, as new species were discovered and added, the listeria genus began to diversify. In 1948, *L. denitrificans* was added, in 1966, *L. grayi* was added in honor of an American microbiologist named Gray, in 1971, *L. murrayi* was added in honor of a Canadian microbiologist (Gray,1966; Murray,1926) *L. welshimeri* was added in 1985 in recognition of American microbiologist Welshimer, and *L. seeligeri* was added in 1983 in recognition of German scientist Seeliger (Ryser,2007).

Listeria species are divided into a number of serotypes, also known as serovariants or serovars, with *L. monocytogenes* comprising serovars (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7). However, practically all infections are caused by certain types, such as 4b, 1/2a, and 1/2b (Hassan, 2007).

2.3 Prevalence of *listeria monocytogenes*

The transmission of listeria in ruminants is unknown, only a few scientists have found a direct link between listerial mastitis in sheep and raw cheese contamination. Sheep suffering from listerial mastitis shed *L. monocytogenes* at high rates over a long period of time without showing any symptoms of disease, posing a potential threat to public health (Schoder *et al*., 2011). *Listeria monocytogenes* was revealed as a common foodborne bacterium that causes listeriosis, which has a 30% mortality rate in susceptible populations like the elderly and debilitated. It is to blame for multiple foodborne listeriosis outbreaks that have been documented in Eu, the United States, and Japan (Schvartzman *et al*., 2011). Many foods especially dairy products, meats, and vegetables, can contain *L. monocytogenes*, and it was spread in nature and may be isolated from a variety of sources, including soil and vegetables, as well as river, channel, and residual water, this was occurred because there is rising concerns about the presence of *L. Monocytogenes* in foods and food processing facilities (Rodas *et al*., 2013).

Raw milk in the production field is one of the most typical ways that *L. monocytogenes* is spread. Listeria spp. with a population of 102 CFU/mL can be eliminated from milk by pasteurization, which takes place at temperatures of 62.8°C for 30 minutes and 71.7 °C for 15 seconds (Kasalica *et al*., 2011). Cheeses that are semi-hard, soft, white, and fresh are also good for growing Listeria, it is possible to commit *L. Monocytogenes* by ingesting handmade cheese made from un pasteurized milk (Şanlibaba,2018). *Listeria monocytogenes* can persist and grow in Processed food in a wide range of temperatures, low pH conditions, and high salt concentrations, therefore the bacteria thought to be a potential cause of contamination and outbreaks in the future (Mansouri-Najand *et al.,* 2015). Listeria has been found in sewage in numerous nations; this presence may have an indirect function by contaminating milk through fodder or silage, for instance, or a direct role by contaminating raw vegetables and possibly fruits (Beuchat,1996).

2.4 Intracellular route of listeriosis

The harsh environment of the stomach must be overcome before the ingested Listeria organisms may enter the intestine. This means that a considerable portion of the Listeria organisms that are ingested with contaminated food may be destroyed by gastric acidity (Schlech *et al.*,1983). Bacteria were primarily found inside macrophages in the stroma of the villi in later phases compared to the absorptive epithelial cells of the early phases. This finding suggests that *L. monocytogenes* enters the host through infecting the intestinal epithelium (Ra´cz, *et al.*,1972).

2.5 Pathogenesis of *listeria monocytogenes*

L. monocytogenes was considered the only the type of Listeria spp. that is pathogenic to both humans and animal species, while the rest of Listeria types are not pathogenic to humans (Cattean, 1995). Motrin, (2004) was mentioned that pathogenicity of the *L. Monocytogenes* in the following stages:

- Attachment of *L. Monocytogenes* to the host cells.
- Escape of *L. Monocytogenes* from the vesicles of the phagocytic cells.
- The bacteria multiply inside the host's cytoplasm.
- Movement of *L. Monocytogenes* within the cytoplasm of the cell and its direction towards the cell surface and the formation of structures similar to false feet, then devouring these structures by neighboring cells and the transfer of the germ to another neighboring cell, and so on.

Linnan *et al*., (1988) were mentioned that following *L. Monocytogenes* consumption, the organism is quickly eliminated from the digestive tract, after an 11–71-day incubation period, *L. Monocytogenes* may result in disease at 31 days, *Listeria Monocytogenes* might continue to exist inside cells, the combination of the three epidemiologic variables—vector, agent, and host, or the amount of the inoculum in contaminated food, the virulence of the organism, and the host risk factors—determines which develops in an individual (Buchholz, 2001). It was believed that the gastrointestinal system serves as the host's main point of entry for pathogenic *Listeria monocytogenes*. In cases of gastroenteritis, the

clinical course of infection often starts about 20 hours after consuming highly contaminated food (Dalton *et al*.,1997).

Similar incubation periods have been recorded in animals for both gastro intestinal and invasive disease, despite the fact that the incubation period for the potential sickness is significantly longer, ranging from 20 to 30 days (vazquez-boland *et al*.,2001). *Listeria monocytogenes* is an intrinsic bacterium that is phagocytosed and internalized in epithelial cells through the interaction between the bacterial surface protein internalin and its receptor on the epithelial surface, E-cadherin. This process occurs after the digestion of infected food (Kuhn, *et al*,2001).

Lorber *et al*., (1990) have been mentioned that macrophages, polymorph nuclear leucocytes, and extra plasma cells vacuolated the organism, and listeriolysin allows it to exit the vacuole. to finally reach the cytoplasm, where proliferative processes take place. Act A, another virulence factor, causes actin polymerization to produce filaments in the cytoplasm, which the organism uses as a force system.

When the organism reaches the cell membrane, it generates pseudopod-like structures that protrude from the host cell. Adjacent cells recognize these structures and phagocytose them to keep the intracellular cycle going (Jack Sobel.,2011). *L. monocytogenes* able to enter the host without compromising the GI tract's integrity. it was proposed that gastrointestinal symptoms could mimic a non-specific reaction to a systemic infection (Kusanovic *et al*, 2011). Memory T-cells provide an acquired resistance to Listeria infection, which explains why listeriosis is linked to malignancy, immunosuppressive therapy, AIDS, pregnancy, and the neonate. Listeria activates T-cell mediated immunity, which, under the influence of cytokines, attracts macrophages that produce inflammatory granulomata where bacteria are damaged (Lamont *et al*.,2011).

2.6 Risk factor of the Host

L. monocytogenes was exposed, host susceptibility has a significant impact on how clinical illness manifests. T-cell-mediated immunity was compromised in the majority of listeriosis patients due to a physiological pathological abnormality. This demonstrates why *L. monocytogenes* should be considered an opportunistic pathogen (Poyart *et al*., 1990). the prognosis of listeriosis was significantly influenced by the patient's state of health. Patients with strong immune systems typically survive listeriosis, whereas those with underlying incapacitating conditions frequently pass away from the infection (Farber *et al*., 1991).

2.7 Distribution of *L. monocytogenes* strains between animal isolates

Listeria Monocytogenes has widespread distribution and frequent occurrence of the majority of Listeria species in various habitats, *L. monocytogenes* was reported around the world and it was isolated throughout, including in North America (Chapin *et al*. 2014) Latin America (Hofer *et al*. 2000) Europe (Gnat *et al*. 2015) and Asia (Huang *et al*., 2015). Cases of animal listeriosis caused by *L. ivanovii* have also been reported on various continents. Most studies that identified reasonable large sets of Listeria isolates that were not *L. monocytogenes* to the species level identified *L. innocua*, *L. seeligeri*, and *L. welshimeri* (Orsi *et al*.,2016).

In previous study to Jeffers *et al*., (2001) were reported in an epidemiological research, same frequencies of listeria isolates were found in fecal samples from case farms but a greater frequency of *L. Monocytogenes* isolates in fecal samples from control farms (farms without cases of ruminant clinical listeriosis). It's interesting to note that in investigations of wild animals, serotype 4b isolates were more common than isolates with other serotypes, Linke *et al*., (2014) were discovered a significant relationship between the presence of Listeria spp. in soil samples and abiotic conditions such as moisture, pH, and soil type. Listeria spp. were more commonly isolated from soil samples with low moisture content, neutral pH, and soil types composed of sand and humus. The same authors discovered a seasonal effect on the prevalence of Listeria spp. in soil, with July having the lowest isolation rates.

2.8 Virulence factors of listeria monocytogenes

Most of the virulence factors identified as being involved in the various steps of the infectious cycle of pathogenic *Listeria monocytogenes* are the major virulence functions of pathogenic *L. monocytogenes* that allow its intracellular life style in hosts (Hassan, 2007). Vasquez *et al*., (2001) were mentioned that numerous virulence factors of *L. monocytogene*s have been demonstrated in studies, and more research is being done to learn more about its virulence and pathogenicity.

2.8.1. Hemolysin

The hemolysin gene (hly) is the first virulence determinant to be identified and sequenced in Listeria Spp. Hly is a key virulence factor having a vital role not only in intracellular parasitism but also in several other functions in the interaction of listeriae with their vertebrate host. The major role of this virulence factor is escaping from phagosome (Vasquez *et al*., 2001). Girard *et al*., (1963) was mentioned when *L. monocytogenes* produced soluble hemolysin for the first time in the 1960 and 1970, a number of studies attempted to purify the substance and describe its poisonous and biochemical characteristics. Jenkins *et al*., (1971) were the first to provide evidence that the Listeria hemolysin is similar in function and antigenicity to streptolysin O (SLO) from Streptococcus pyogenes, Hemolysin suppressed by cholesterol, its optimum pH was less than 7, and it had cytotoxic properties in phagocytic cells. They were also the first to propose that hemolysin might be involved in phagosomal membrane disruption. (Vasquez *et al*., 2001).

2.8.2 Phospholipase

Patrick *et al*., (2001) was mentioned that three distinct phospholipase C (PLC)-active enzymes important in virulence are produced by pathogenic Listeria spp. PlcA and PlcB are two that were found in both *L. monocytogenes* and *L. ivanovii*. Fuzi and Pillis, (1962) were identified *L. Monocytogenes* produces phospholipase activity which consists of two

different types: phospholipase specific to phosphatidylinositol (PI-PLS) and phosphatidylcholine (PC-PLS), which cooperate to allow the germ to enter the host cells as well as to travel from one cell to another. Marquis *et al.*, (1995) have been showed that each type of *Listeria monocytogenes* has a biological function, as the enzyme PI-PLS helps to get rid of the process of phagocytosis by breaking down the fat of the membrane and thus making openings that help in getting rid of the process of phagocytosis, while the enzyme PC-PLS helps in the transmission of the pathogen from one cell to another in the host's body.

2.8.3 Actin A

The study of pathogenic Listeria Spp. Actin-based intracellular motility has received a lot of attention in recent years (Beckerle, 1998). The unique behavior of an isogenic act A mutant of L. monocytogenes in infected tissue culture cells served as the first indication of the crucial function of act A in listerial intracellular movement and virulence (Kocks *et al.*,1992). Act A consists of 610 amino acids, act A protein consists of three functional parts adapted from (Losa *et al.*, 1997):

1. The N-terminal Region.
2. Central Proline Rich Region.
3. C-Terminal Region.

The N-region and the central region are necessary for the movement of the bacteria from cell to another in the host body as well as for actin polymerization (Pistor *et al.*, 1995). Moors *et al.*, (1999) were that where the polymerization of globular actin molecules (G-actin) is stimulated and converted into actin filaments, in addition, these sutures form protrusions in the membrane, forming structures similar to pseudopod-like structures, then the bacterium begins to move on the length of these structures and thus Act A is responsible for the movement of the bacteria. Pirie, (1940) was explained that the movement is regulated by a group of Phosphoinositide, especially Bisphosphate and Phosphatidylinositol. Act A is not found in the actin tail, indicating that the listeria surface protein is not necessary for stabilizing the actin mesh work that is produced when moving Listeria cells (Kocks, 1992).

2.8.4 Internalins

Internalins considered as protein derivatives of genes associated with virulence factors found in pathogenic Listeria spp. It is also one of the first members of this family to be identified (Gaillard *et al.,*1991). Iqbal, (2005) was mentioned that internalin aids *L. Monocytogenes* penetration into host cells and stimulates unspecialized cells such as epithelial and endothelial cells for phagocytosis, internalins divides into two parts: Inl A and Inl B.

2.8.4.1 Internalin A Protein (Int A)

Consisting of 710 amino acids, this protein aids in the penetration of L.M into cells that are not specialized for phagocytosis (Gaillard *et al*., 1991). Kuhn *et al*., (2001) Int A on the surface of the bacteria cells binds to a surface protein called E-Cadherin on the surface of epithelial cells of the host, and this receptor is found on Hepatocytes, brain endothelial cells, intestinal epithelial cells as well as placental villi, this association stimulates phagocytosis process, after phagocytosis, LLO is produced, which has the ability to break down the wall of the cell and the germ is discharged into the host cells' cytoplasm.

2.8.4.2 Internalin B Protein (Int B)

Drams *et al*., (1995) were mentioned this protein plays a role in the penetration of the bacteria into the hepatocytes cell.

2.8.5 P104 protein

This protein was recently discovered, and it has a great role in the process of adhesion to cells in host (Pandiripally *et al*., 1999).

2.8.6 P60 protein

It is a protein found in all species of the genus Listeria, but it differs in the amino acid sequence from one species to another, and it can be distinguished by PCR and immunological detection methods (Bubert *et al*., 1994). It was found that the gene for this protein has a role in the process of phagocytosis of the bacteria cell (Kuhn *et al*., 1999). Many studies have been indicated the role of P60 in penetrating the bacterium into the intestinal wall and its resistance to the intestinal periphery (Hess *et al*., 1995).

Geginat *et al*., (1998) noted that P60 is the main antigen in body protection. Against *L. monocytogenes*, which can be used as a vaccine in the future.

2.8.7 Enzymes

Iqbal, (2005) was mentioned that there are two important types of *L. Monocytogenes* enzymes: ATPase and CL Protease (CLP). *L.Monocytogenes* resists environmental conditions such as temperature, pH and osmotic pressure with the help of enzymes produced by the bacterium, namely CLP and Atpase, which have a role in the pathogenesis of the bacterium. CLP is a casein-dissolving enzyme and is divided into several types:

1. CLP C: Act A protein helps in the transmission of bacteria from one cell to another, in addition to helping the ATPase enzyme in pathogenesis (Nair *et al*., 2000).

2. CLP E: ATPase helps with pathogenesis, and works synergistically with

CLP C (Nair *et al*., 2000).

3. CLP P: it is molecular weight is 21.6 Kda, and it helps the microbe to resist environmental conditions as well as it helps to get rid of the phagocytosis process (LLO) (Gaillot *et al*., 2000).

Olivier *et al*., (2001) were found in laboratory animal experiments that when mice were injected with a high dose of dead Listeria Monocytogenes carrying CLP P, it stimulated the production of LLO toxin in a large amount, and thus it became immune to the germ.

2.8.8 Other virulence factors

Listeria Monocytogenes proteins that have been examined so far are real virulence factors since they have only ever evolved in pathogenic Listeria species and predominantly carry out actions that are necessary for parasitizing vertebrate hosts (Kuhn *et al*.,2001).

Bereche *et al*., (2001) was mentioned that other listerial products have been found in addition to these virulence factors aid in the organism's survival inside the host. Even while some of them have a significant impact on how the host-parasite interaction plays out their involvement in pathogenesis is more indirect because they are likely engaged in general housekeeping tasks that are also essential for saprophytic existence.

2.8.8.1. Antioxidant factors

Following immunological activation by cytokines like IFN-g or by binding of complement receptor 3 during C3b-facilitated opsonic phagocytosis of L. monocytogenes, macrophages become listericidal (Campbell, 1993). Beckerman *et al*., (1993) were revealed the exact mechanism by which macrophages eliminate intracellular parasites like *L. monocytogenes* is unknown. it is believed that the production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) during the oxidative burst plays a crucial role.

2.8.8.2 Metal iron uptake

Iron is not readily available in animal host tissues because it is bound by ferric transferrin in serum and ferritin and hem compounds in cells as a result. Bacterial pathogens have developed unique mechanisms to get iron for growth in host tissues, and these processes are crucial for virulence (Payne, 1993). Stelma *et al*.,)1987 (were mentioned that iron not only stimulates growth in synthetic medium in *L. monocytogenes*, but when administered in salt form to infected mice. It also increases bacterial proliferation rates in the liver and spleen and decreases the LD50.

2.8.8.3 Stress response mediators

De chastellier *et al*., (1994) were mentioned that small percentage of *L. monocytogenes* cells manage to escape the harsh, stressful phagocytic vacuole and make it to the cytoplasm, where they multiply and disseminate the infection. A group of conserved proteins that are increased in vitro in response to heat shock, low pH, reactive agents, toxic

chemical compounds, hunger, and, generally, any condition in which bacterial growth is inhibited is necessary for survival under stress.

2.9 Significance of listeria monocytogenes

listeria monocytogenes could persist in soil for several months, most disinfectants and pasteurization reject the organism (Sarah,2018). *listeria monocytogenes* was intracellular bacterial organism that can cause a severe intrusive disease (listeriosis) mainly in immune suppressed, aged individuals, and gravid women, distinguished by sepsis, inflammation of meninges and abortion (Qassim *et al*., 2017). Milk and milk products seems to be susceptible to contamination, the prevalence of Listeria ssp. varies according to season, and the bacteria ratio in raw milk usually is <1bacterium/mi, Heat treatment considerably reduces the concentration of the bacteria so that pasteurized milk does not present significantly risk to the health of individuals (Sanaa *et al*,1993).

2.10 *Listeria monocytogenes* presence in milk and milk products

Raw milk, soft cheese, ice-cream, yoghurt is the most dangerous food contaminated with this bacterium, this depends on the source of the germ and the ability of the milk or product type to preserve the germ and the occurrence of contamination after different treatments of milk and its products or as a result of contamination where the source of listeria is the animal that produces the milk itself, directly or indirectly environment, milkers and other sources (Robinson,2002). The ability of the product to preserve germs, it depends on the vital factors it contains that help in the growth or inhibition of bacteria, such as pH, salinity, water efficiency and the extent of its exposure to efficient thermal treatments such as pasteurization and others, especially soft cheeses made from raw milk and other unpasteurized product (Alshamary,2010).

2.11 Biochemical specification of listeria

Colony of *Listeria Monocytogenes* have been revealed as β hemolysis on sheep blood agar, camp test and acid production from short range of sugars are useful methods to differentiate listeria species in laboratory the bacteria colonies appear tiny, smooth, and translucent (Quinn,2002). listeria strains grow on glucose, forming lactate, acetate, and all strains are methyl red, Hydrogen sulfide (H_2S) is not produced. urea is not hydrolyzed and indole is not produced. Listeria species are phenotypically very identical but can be differentiate by many tests (Hassan, 2007).

Table (2-1) The following laboratory methods for distinguishing listeria species which adapted from (Quinn,2002).

Species of listeria	Hemolysis on sheep blood agar	Camp test		Acid production from sugars		
		s. aureus	R. equi	D-mannitol	L-rhamnose	D-xylose
L.Monocytogenes	+	+	-	-	+	-
L.ivanovii	++	-	+	-	-	+
L.seeligeri	+	+	-	-	-	+
L.innocua	-	-	-	-	v	-
L.welshimeri	-	-	-	-	v	+
L.grayi	-	-	-	+	v	-

2.12 Antimicrobial susceptibility of listeria monocytogenes

Antibiotics have successfully been used to treat human listeriosis for decades. However, antibiotic-resistant strains of *L. monocytogenes* were emerged (Abdollahzadeh,2016). The vast prescription of antibiotics in human listeriosis and the livestock industry have raised the drug resistance of *L. Monocytogenes.*

L. monocytogenes basically becomes resistant to antimicrobial agents through the acquisition of genetic elements, such as plasmids, transposons, and integrons. Environmental stresses such as osmotic, acidity, oxidative, and cold also contribute to the increase of antimicrobial resistance of *L. monocytogenes* (Erdem, 2010).

Several mechanisms cause antibiotic resistance in this bacterium such as inhibition of cell membrane function, changes in the protein and nucleic acid synthesis, mutations in bacterial genes, and the most importantly, activation of efflux pumps (Noll, 2018). According to the research data, the acquisition of plasmids and transposons from streptococci and enterococci evolves the drug-resistant strains of *L. monocytogenes*. The human isolates of *L. monocytogenes* have been susceptible to several antibiotics such as penicillins, aminoglycosides, macrolide, tetracycline, rifampin, Co-trimoxazole, vancomycin, and carbapenems. Nevertheless, this bacterium is intrinsically resistant to fluoroquinolones, lincosamides, and cephalosporins, especially third and fourth generations (De Noordhout,2014).

Penicillins and aminoglycosides (such as gentamicin) are the first-line treatment for infections caused by *L. monocytogenes* other antibiotics are considered as the alternative treatment
especially for pregnant women and patients with allergies to penicillins (Rostamian,2022).

2.13 Resistance to stress conditions

Temperature regulation as well as thermal treatments have been used in food processing and handling for centuries to prevent or limit contamination and the spread of food-borne pathogenic organisms. Furthermore, the efficacy of thermal treatments against *L. monocytogenes* is limited by the pathogen's inherent ability to survive and actively replicate at temperatures ranging from 0.4 C° to 45C° (Chan *et al*.,2009). In the event of such a heat shock microbial cells containing these stress proteins may be more resistant to future severe stresses. Thus, cellular adaptation to one stress may shield them from subsequent, extremely severe homologous or heterologous stress (Lou *et al*.,1996).

2.13.1 Resistance to thermal treatment

The ability of *L. Monocytogenes* to endure or even reproduce in a variety of environmental stress situations is well documented (Gahan and Hill., 2014). Sallami *et al*., (2006) were mentioned that in spite of *L. monocytogenes* does not indicate extreme tolerance to high temperatures, it has been demonstrated that it is more heat tolerant than other non-spore-forming pathogens like Salmonella and E. coli. In the instance of milk obtained from purposefully contaminated cows, *L. monocytogenes* has been demonstrated to withstand the minimum high-temperature, brief-time treatment required by the United States Food and drug administration 71.7°C, 15seconds (Bucur *et al*.,2018). Early research suggested that *L. monocytogenes* living inside these cells during heat treatments might be protected by polymorph nuclear leukocytes found in milk (Fleming *et al*., 1985). But further studies revealed that *L. monocytogenes* could not survive

temperatures beyond 67.5oC and a holding period of 16.2 s in naturally contaminated milk (Farber *et al*., 1988).

2.13.2 Resistance to cold stress

The tolerance to cold stress is responsible for the frequent detection of *L. monocytogenes* in refrigerated food products especially meat, milk, poultry, and seafood (Tasara, T., & Stephan, R, 2006).

Low temperatures result in decreased metabolic rates and changes in membrane composition, the alterations in the membrane in response to cold stress comprise a reduced chain length of fatty acids, an increase in the concentration of unsaturated fatty acids, and altered ratios of iso and ante iso-branched fatty acids (Iqbal,2005).

2.13.3 Resistance to UV radiation

Kasalica *et al*., (2011) mentioned that *L. Monocytogenes* is durable and Resistant radiation, which significantly aids in the bacterium's wide spread. Studies support its resistance to X and gamma radiation. *L. Monocytogenes* (104/ml) is fully destroyed by doses of 2.6 kGy in milk, but at a concentration of 105/ml, some pathogens can survive for 45 days even if they are no longer able to replicate.

2.13.4 Resistance to sanitation and chemicals

Most sanitation preparations, chemicals are ineffective against *L. monocytogenes*; however, the following preparations have shown to be the most effective at eliminating *L. monocytogenes* formulations based on solutions of chlorine, quaternary ammonium compounds, and iodoform per acetic and peroctanoic acid (Eifert and sanglay ., 2002).

2.14 Molecular Detection of listeria species

The development and use of several PCR-based approaches, including conventional PCR, and their applications in the detection and characterization of L. monocytogenes, as well as classic culture-based procedures, are described (Chen *et al*.,2017).

Borucki M., (2003) was mentioned that *Listeria monocytogenes* serotyping is a widely used subtyping technique (gold standard) for comparing isolates tested in various labs and using various procedures is provided by the determination of the strain serotype, which enables distinguishing between significant food-borne strains (1/2a, 1/2b, and 4b). This identification system would become much more simple and available with the identification of PCR serotyping primers. Variable sections of the *L. monocytogenes* genome were used to construct PCR primers for serotyping (Douglas, 2003).

Several PCR-based methods such as restriction enzyme analysis-PCR, PCR-single-strand conformation polymorphism, and mismatch amplification mutation assays-PCR were described for rapid L. monocytogenes interspecies subdivision typing (Doumith *et al*.,2004). The most widely used ELISA techniques are those that employ an antibody immobilized to a microtitre well for antigen capture, along with a secondary antibody coupled to an enzyme (or some other tag) to detect the captured antigen. These techniques combigne simplicity of use with the production of quick test results (Denise *et al*.,2004). A technological invention is the availability of "next day" Listeria tests for food samples. ELISA technique may be employed with challenging sample matrices, making these tests particularly well suited for food testing (S. kusanovic *et al*.,2011).

2.14.1 Detection of listeriosis in animals

Almost all domestic animals, including sheep (Ward rope and Macleod, N.S, 1983), cattle (Wesley,1999) goats (Sharma *et al*., 1983) and less frequently birds (Ramos *et al*., 2019) are susceptible to listeria infection. Healthy asymptomatic animals shed a significant amount of *L. monocytogenes* in their feces (Ward rope *et al*.,1983, Rebhun *et al*.,1982, Sharma *et al*.,1983 and Ramos *et al*.,2019). Despite the fact that the majority of infections are subclinical listeriosis in animals can be sporadic or epidemic in nature and frequently results in encephalitis that is lethal. In livestock, clinical listeriosis manifests as encephalitis, septicemia, and abortions in the third trimester of pregnancy (Hassan, 2007).

Flaming (1985) mentioned that there are many foods that have a role in the disease outbreaks in listeriosis, which are fresh vegetables, milk, cheese and meat, especially poultry meat.

Sun Young *et al*., (1999) found that in Korea isolation of the *L. Monocytogenes* from foods Frozen, beef, poultry, fresh milk and ice cream in the following proportions 9.8% ، 3% ، 26% ، 2% ، 8% respectively. also listeriosis cases that occurred in Mexico were caused by milk from cows infected with *L. monocytogenes*.

Cases of infection were recorded in North America and Europe in 1983 caused by raw milk (Mclanchlin, 1987). Bushan (2002) was mentioned that infection with listeriosis causes symptoms identical to those of food poisoning, such as diarrhea, increase in temperature and nausea, but this case is not common and this may be attributed to the incubation period of *L. Monocytogenes* which reaches from (1-90) days, therefore it is difficult to determine the infection. Food contaminate with *L. monocytogenes* during treatment processes especially milk (Lovett, 1989) cheese (Silva *et al*, 1998). Schlech, (1983) mentioned that refrigerated foods occupy a high percentage among the foods that cause listeriosis cases, and this is attributed to the fact that the bacterium *L. monocytogenes* resists refrigeration temperature.

2.14.2 Detection of listeria in human:

According to Murray *et al*. (1926), the digestive tract may be the point of entry for microorganisms that can cause Listeria infections. Most investigations of nasopharyngeal isolates from healthy people failed to find Listeria spp. The discovery of *L. Monocytogenes* in the feces of healthy individuals by numerous researchers supports the idea that the gastrointestinal tract serves as the organism's human reservoir (Schuchat *et al*.,1992). In diverse nations, the prevalence of listeriosis ranges from 0.1 to 11.3/1,000,000, and the majority of recorded cases manifest as a potentially fatal sickness in one of three clinical syndromes meningoencephalitis, blood stream illness, or maternal-fetal or neonatal listeriosis. The usual case fatality rate for listeriosis is 20–30% despite effective antibiotic treatment (Rocourt *et al*,.2003). Consequently, a focal infection caused by listeriosis may appear.

2.15 Microscopic characteristics of *L. monocytogenes*

Listeria species are small rods that are facultative anaerobic, Gram positive, do not produce spores, lacking capsules, and are organized separately or in short series on a periodic basis in the shapes of the letters V and Y (Kasalica *et al*.,2011). Sometimes they appear as bacilli in smear stained with gram stain obtained from solid culture media, motile in 25°C its motion appears in liquid media in the form of tumbling movement, in semisolid media it appear as umbrella shape, grow with different types of colony and produce tight zone of blood hemolysis (Iqbal ,2005).

2.16 Culture features of *listeria monocytogenes*

Listeria monocytogenes called super bacterium because of its ability to resist environmental conditions, the optimum degree of its growth 37°C, Resistant to heat and relatively drought, as it remains alive at 60°C for 10 minutes, it’s also resist pasteurization at 72.2 °C for 15 minutes since the bacteria intracellular organism, Bacteria also have a special property of growing at a cooling temperature of 4°C (Norman, 1999). Geo *et al*, (2001) were reported that the best isolation rate of the germ, especially from the tissue, can be obtained when incubating it for several days at a temperature of 4 °C before inoculating it on the culture media, which facilitates its isolation.

Listeria monocytogenes was beard different growth conditions such as growth in a medium with pH ranges between 5.5-9.6 and at a high concentration of salinity up to more than 10% sodium chloride in addition to lithium chloride, acriflavin, Potassium Tellurite, these materials are used as electoral agents in culture media for isolating and growing the germ, listeria is aerobic bacteria and it can grow in a proportion of CO2 up to 10%, as well as its ability to dissolve ascoline (Mclain & Lee, 1988).

Listeria monocytogenes was grown on a blood agar medium, and its colonies are characterized by their very small size, and are described as being as large as a pin point, the colonies are smooth, translucent, raised from the center, completely (β-Hemolysis) especially pathogenic ones when grown on media prepared from the blood of sheep, horses and humans. listeria also grow on rich media such as trypticase soy agar, trypton soy agar, oxford agar and palcam agar (Iqbal,2005). The colonies of *L. monocytogenes*

developed on nutritional agar are smooth, punctate, bluish gray, transparent, slightly elevated, with a fine surface texture and complete edge, and they range in size from 0.2 to 0.8 mm. colonies that have been well-separated for 5 to 10 days may have a diameter of at least 5 mm, If *L. monocytogenes* colonies grown on trypticase soy agar (TSA) (Clear medium) exhibit a distinctive blue-green shine by obliquely transmitted light, this characteristic is important identify of *Monocytogenes* (Hassan Al-Gara'awi, 2007).

2.17 Hygiene measures

Concentrating on and increasing hygiene in dairy production facilities is essential to preventing *L. monocytogenes* contamination of milk and its derivatives, washing with detergent and properly disinfecting clean surfaces are sanitation practices, every dairy must have a sewage frequency plan that specifies a precise schedule for cleaning and sanitizing the facility's machinery, flooring, drainpipes, walls, cold storage, etc.

Recognizing that sanitizers should be replaced occasionally as they are there is evidence to support the idea that *L. monocytogenes* develops resistance to some medications over time (Kasalica *et al*., 2011).

Chemical solutions It is thought to be the most effective in this approach against *L. monocytogenes* Iodoform, quaternary ammonium compounds, peroxytic and pyroctanoic acids, and solutions based on chlorine dioxide are used to clean equipment. Idoform-based preparations are advised for use in the dairy industry because their remnants do not disturb the starting cultures (Vuković *et al*.,2011).

Memiši *et al*., (2011) were recommended that quarterly ammonium chemicals are not advised for use directly on food-contact surfaces since even minute residues of these substances can contaminate food, Starter farms' operations are disrupted by the compounds, but they are quite good at cleaning them drains, walls, cold storage, and floors.

2.18 Commercial sanitizers on inactivation of *Listeria monocytogenes*

In the food industry, the use of sanitizers and cleaners has been incorporated into good manufacturing practices regimes to prevent the accumulation of microbial cells and consequent biofilm formation (Hood & Zottola, 1995). However, various sanitizers extensively used by food processors may not be very effective against some bacterial biofilms, and alternative removal strategies have been studied. Because of innate differences in antimicrobial susceptibilities, and the altered physiological state of some cells in biofilms, the effectiveness of industrial sanitizers may be affected. A safe and effective sanitization process should ensure an acceptable reduction in microbial levels without the presence of toxic residuals (D'Angelis, & De Martinis, 2008).

Methods have been developed to evaluate the inactivation efficacy of sanitizers against biofilm bacteria using various substrate materials such as stainless steel, rubber, and polystyrene coupons (Medeiros & Sofos, 2009).

However, these methods are time-consuming and are unlikely to produce biofilms with consistent numbers of bacterial cells for comparison. Moreover, these methods do not allow easy testing of a variety of sanitizer products, a wide range of concentrations or different bacterial strains. A polyvinyl chloride (PVC) microtiter plate assay has been used to evaluate the biofilm formation of *L. monocytogenes* and it was considered to be a rapid and simple method to screen for differences in biofilm production between strains (Cruz, C. D., & Fletcher, G. C.2012).

2.19 Public health consideration

Listeria spp. contaminate milk after pasteurization results in high numbers of the organism in the product even though the milk was properly refrigerated after contamination (Northolt *et al.*,1988). Investigated the roles of *L. monocytogenes* in intensely pasteurized milk and high-temperature short-time (HTST)-pasteurized milk. *L. monocytogenes* were less prevalent over the first two days of storage.

Bactericidal components of milk including lacto peroxidase, lysozyme, lactoferrin, and agglutinins may be responsible for this lag phase, these compounds have been demonstrated to limit *L. monocytogenes*' ability to thrive in milk, although rigorous

pasteurization prevented this lag phase from happening. Later, *L. monocytogenes* in pasteurized skim milk may reach dangerously high levels (Farber *et al*., 1992).

Rose now and Marth (1987) were estimated that *L. monocytogenes* doubling times in pasteurized milks range from about 30 to 36 hours at 4°C, though much faster growth has been reported by other researchers. When milk is stored at 4°C, a population of 10 *L. monocytogenes* /l can reach >6000 CFU/1 in 14 days. The infectious dose of *L. monocytogenes* is unknown, but it is thought to be in the range of 100 to 1000 cells. As a result, low initial counts can accumulate to potentially dangerous levels during milk refrigerated storage. Marshall and schmidt (1988) were shown that other prevalent psychrotrophic bacteria like Pseudomonas spp. did not suffer from *L. monocytogenes*' presence in milk, however, *L. monocytogenes* grew more quickly in milks preincubated with Pseudomonas spp. than when it was incubated by itself.

Farber *et al*.,1991 were found that The number of *L. monocytogenes* in butter made from contaminated cream increased by at least two orders of magnitude during refrigerated storage. However, cases of illness involving butter as the vehicle for spread are uncommon, and workers were unable to isolate *L. monocytogenes* from this source. El-Kest and Marth (1992) showed that *L. Monocytogenes* can survive in ice cream mixes, and recalls of ice cream and other frozen desserts have been issued because of contamination with the organism.

Champagne *et al*., (1994) were reviewed the effect of freezing on a number of bacteria including *L. monocytogenes* and the organism can survive freezing at -18°C for 14 d, there remains some concern about the efficacy of current pasteurization procedures applied to viscous products.

Chapter Three: Methodology

3. Materials and Methods

3.1 Materials

3.1.1 Equipments and Instruments

The equipment used through this study were listed in **table (3-1)**

No.	Equipments	Origin
1	Bunsen burner	Shndon/England
2	Beakers	Iwaki glass/Japan
3	Conical flask	Marienfeld/Germany
4	Distillatory apparatus	AlabTech /Korea
5	Disposable syringe	Changazhou medical appliances/China
6	Disposable plastic cup (50 ml)	
7	Disposable tips	CAPP/Denmark
8	Disposable tips with filter	Bioneer/Korea
9	Disposable gloves without powder	Bioneer/Korea
10	Eppendrof tube (1.5)ml	Heittch/Germany
11	Eppendrof rack	Eppendrof/Germany
12	Loop	Shndon/England
13	Micropipettes	Slamid/Germany
14	Petri dishes Plates	Sterilin/England
15	Slides	Sail Brand/China
16	Sterile Mask	Bioneer/Korea
17	Swabs	Arth Al-Rafidain/China
18	Test tubes	Arth Al-Rafidain/China

Table (3-2): The instruments and their origin.

No.	Instruments	Origin
1	Autoclave	Hirayamy/Japan
2	Centrifuge	Hettich/Germany
3	Electrophoresis apparatus	Bio-Rad/Italy
4	Hood	LabTech/ Korea
5	Hot plate	
6	Incubator	Memmert/ Germany
7	Light microscope	Human/Germany
8	Nano drop	Thermo Scientific/ UK
9	Oven	Hirayama /Japan
10	Refrigerator	LG /Korea
11	Sensitive balance	Sartorius /Germany
12	Thermo cycler	Syngene/ England
13	UV- Trans illuminator	Stuart/UK
14	Vortex mixer	Memmert /Germany
15	Water bath	Techen/ England
16	Digital Camera	Canon / Japan
17	Incubator	Memmert / Germany
18	Micro centrifuge (1.5 ml tube)	Hettich / Germany
19	pH Meter	Radio meter / Denmark

3.1.2 Chemical Materials

Chemical materials used in this study were listed in **Table (2-3)**

No.	Chemical	Origin
1	Agarose	BDH/ England
2	Silver nitrate	BDH/ England
3	potassium chromate	Syrbio/Switzerland
4	sodium hydroxide	BDH/ England
5	Hydrogen peroxide (H2O2) 70%	SDI (Iraq)
6	Gram Stain	Drugs and medical appliances/Iraq
7	Normal saline	Haidylena/Egypt
8	Oil immersion	BDH/UK

Table (3-3): Chemical materials

(3-4) Culture media used in this study

Medium	Origin
Oxford agar	United state
Muller Hinton agar	Syrbio/Switzerland

3.1.3. Culture Media

listeria selective agar (oxford formulation) oxford agar is a selective and diagnostic media for the detection of Listeria monocytogenes, when prepared from Listeria Selective Agar Base and Listeria Selective Supplement SR0140 or Modified Listeria Selective Supplement (Oxford) SR0206. Curtis *et al*., (1989) have been mentioned that Listeria Selective Medium (Oxford Formulation) recommended for the detection of *Listeria monocytogenes* from clinical samples and food specimens.

Table (3-5) *listeria* selective supplement (oxford formulation)

Vial contents (each vial is sufficient for500ml of medium)	per vial	per liter
Cycloheximide	200mg	400mg
Colistin sulphate	10.0mg	20.0mg
Acriflavin	2.5mg	5.0mg
Cefotetan	1.0mg	2.0mg
Fosfomycin	5.0mg	10.0mg

The medium utilizes:

(i) the selective inhibitory components lithium chloride, acriflavin, colistin sulphate, cefotetan, cycloheximide or amphotericin B and fosfomycin,

(ii) the indicator system aesculin and ferrous iron for the isolation or differentiation of Listeria monocytogenes.

3.1.4 Amplification PCR Primers

Single-stranded DNA oligo's with a length of about 20 nucleotides are used as PCR primers. In PCR, they serve as the initial step in copying or amplifying. Each PCR reaction uses two primers to surround the target area of copying. In addition to providing a "free" 3'-OH group for the DNA polymerase to add dNTPs to, they bind to the template DNA's opposing strands. http://genolist.pasteur.fr/ListiList/index.html. Primers for conventional PCR were obtained from (Eurogentec Aid. Belgium). The hemolysin (hly) primers and list primers were designed according to the references mentioned in table (3-5).

Table (3-6): Primers used in this study

Species	target gene	Primer sequence (5'to3')	Size	Reference
L.Monocytogenes	List-f	5'-GGACCGGGGCTAATACCGAATGATAA-3' (26mer)	1100 pb	(Sue *et al.*, 2004).
	List_ R	5'-TTCATGTAGGCGAGTTGCAGCCTA-3' (24mer)		
	Hly_1_F	5'-ATTTTCCCTTCACTGATTGC-3' (20mer)	250 pb	(Chaturongakul *et al.*, 2006)
	Hly_2_R	5'-CACTCAGCATTGATTTGCCA-3'(20mer)		

F; Forward Primer **R; Reverse Primer** **Amplicon size; PCR product size**

3.1.5 Stains and Solutions Preparation

3.1.5.1 Gram Stain Solution

Gram staining is essential for phenotypic characterization of bacteria; the staining procedure distinguishes Bacteria organisms based on cell wall structure. Gram stain solution consist of four agents:

1. Primary Stain (Crystal Violet Staining Reagent)

2. Counterstain (Safranin)

3. Decolorizing Agent

4. Grams iodine solution

PROTOCOL

According to (Gerhardt *et al*, 1981)

1. Crystal violet staining reagent was applied to an air-dried, heat-fixed cell smear for 1 minute. The Gram Stain results will be affected by the smear quality (too heavy or too light cell concentration).

2. Slide should be washed for two seconds in a mild, indirect stream of water.

3. flood the slide with Gram's iodine, for 1 minute.

4. Wash the slide in a gentle, indirect stream of tap water, for 2 seconds.

5. Decolorizing agent should be applied to the slide drop by drop for 15 seconds or until the decolorizing agent runs clean.

6. Overflow the slide with safranin counterstain, between 30 seconds and 1 minute.

7. Slide should be washed with a gentle, indirect stream of tap water until the effluent is colorless, and then it should be blotted dry with absorbent paper.

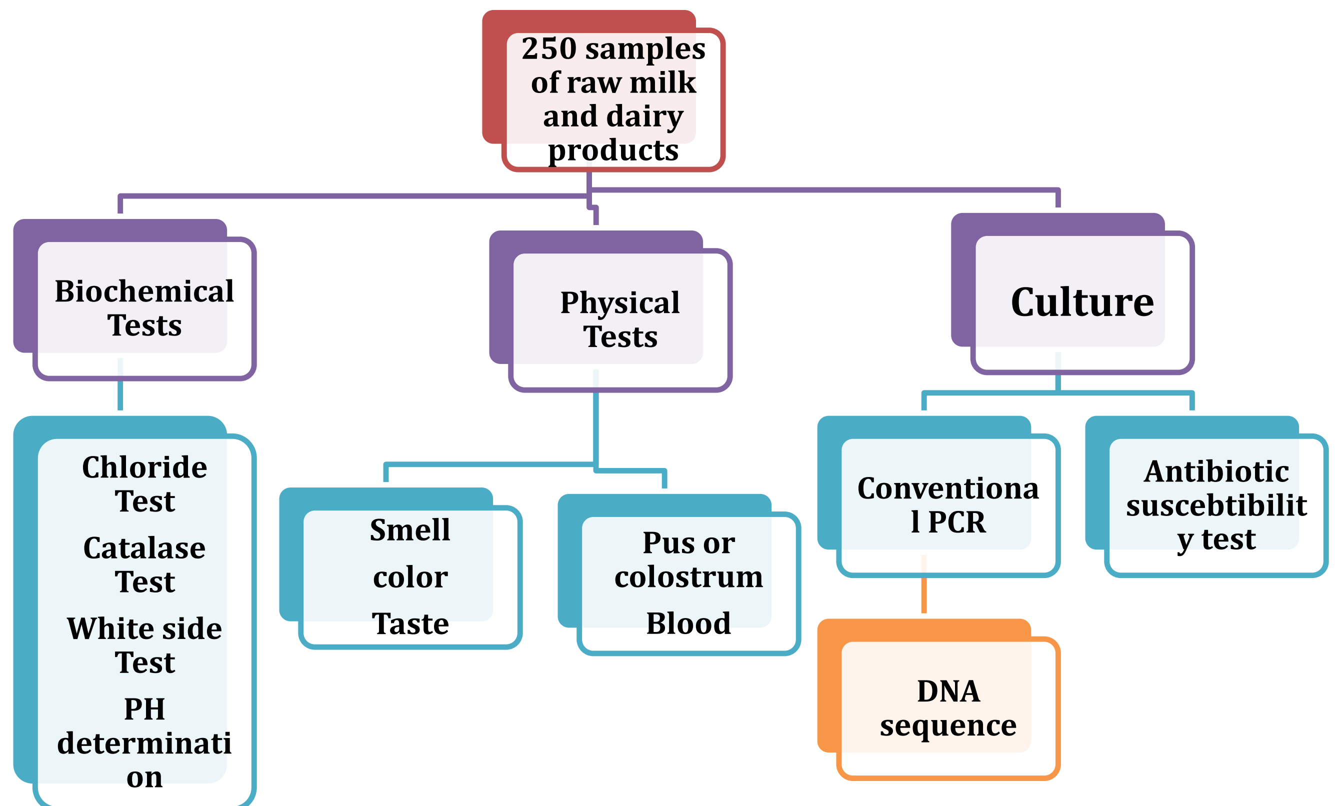
8. Using a Bright field microscope, examine the results of the staining procedure under oil immersion. Gram-negative bacteria will stain pink to red when the Gram Stain is finished, while gram-positive bacteria will stain blue to purple.

3.1.5.2 Catalase Reagent

3.2 Methods

3.2.1 Study Design

Figure (3-1): Study Design



3.2.2 The animal

The case history of 250 Cow from farms and livestock that suspect of infecting with *Listeria Monocytogenes* was taken, Samples of raw milk and dairy products were preferred for taken from animals with previous abortion cases, immunosuppressed and depressed cows, general examination of the udder and teats were done.

3.2.3 collection of samples from raw milk and milk products

Over the course of four months, 250 samples of raw milk and dairy products were gathered from various districts and local markets in Karbala City and tested for L. monocytogenes presence in local milk samples which were: Farm-raised raw milk (50 samples) market-fresh milk (25 samples) raw milk from various sell points (25 samples) and 150 samples of milk products (soft cheese, yoghurt, butter) were collected under sterile and cooled condition

during 2022 and transferred to bacteriological laboratory to perform the tests and examination.

3.3 Isolation of listeria species

3.3.1 isolation agar

The Oxford Agar is based on the Curtis *et al*., (1989) formulation for Listeria monocytogenes isolation. Special peptone supplies minerals, vitamins, and nitrogen. The absence of corn starch lowers opalescence. Osmotic equilibrium is secured by sodium chloride. Gram-negative bacteria and the majority of gram-positive bacteria are inhibited from growing when lithium chloride and antibiotics such acriflavin, colistin sulfate, cefotetan, cefotaxim, cycloheximide, and fosfomycin are added. Because Listeria has a high tolerance to salt, lithium chloride provides a modest level of selectivity, the ingredients in the Oxford Listeria Selective Supplement are antibiotics. Some specific Esculin-negative colonies of Staphylococci strains can develop. Listeria monocytogenes hydrolyses esculin to esculetin and forms a black complex with iron(lll) ions.

Procedure

Dissolve 27.8 g in 500 ml distilled water and mix thoroughly, bring to the boil by gently heating Autoclave at 121°C for 15 minutes and cool to 45-50°C. Aseptically add 1 vial of sterile supplement Mix well and distribute.

3.3.2 Isolation procedure

The ISO 11290 method was used to isolate the Listeria monocytogenes. a 25 ml sample was pre-enriched in 225 ml of half-strength Fraser broth containing selective supplements (Hi Media) for 24 hours at 30°C. then, 0.1 ml of the pre-enriched Fraser broth content was then further enriched in 10 ml of full-strength Fraser broth containing selective supplements (Hi Media) for 48 hours at 37°C. The inoculum was plated on Oxford agar (Hi Media) following the enrichment process, and it was then incubated for 48 hours at 37°C (Curtis *et al*., 1989).

3.4 Physical and Biochemical tests

The physical and chemical tests have been conducted according to (Najim *et al*., 2012).

3.4.1 Physical tests

Physical methods used to detect low quality milk, testing milk for organoleptic characteristics Also called sensory evaluation testing, and uses the normal sense of sight, smell and taste to determine the overall quality by inspecting the lid and container for visible dirt and impurities, pus or colostrum, blood, Abnormal flavors, Bad smell or taste of milk may be due to bacteria, chemical reactions or by absorbed flavors. high-quality raw milk must be clear of debris and silt, free of off flavors, normal in composition and pH, low in germs, and devoid of additives (such as antibiotics and detergents). The main element affecting the quality of milk products is the quality of the raw milk. Only high-quality raw milk can be used to make milk products. the production of milk and milk products that are secure and appropriate for their intended applications depends critically on the sanitary quality of the milk. The dairy supply chain as a whole need to adhere to appropriate sanitary standards to achieve this quality. The informal and uncontrolled marketing, handling, and processing of dairy products; a lack of funding are some of the factors that small-scale dairy producers face while trying to manufacture hygienic goods.

The normal physical features of raw cow milk including (color, taste, smell) which mentioned by (Najim *et al*., 2012).

Table (3-7) physical features of raw cow milk

	Features of normal milk		
	Color	**Taste**	**Smell**
Milk	white to creamy white, slightly yellow	Slightly, sweet flavor	little distinct odor
Cheese	Deep yellow/straw colored	rich and buttery	sour milk
Yogurt	White	slightly sour	fresh, or barely
Butter	Pale yellow	soft, rich, with just a hint of sweetness.	cheese-like smell.

3.4.2 chemical tests

3.4.2.1 pH determination

The amount of lactic acid produced by microbial activity is measured by the pH of milk. The higher the acidity, the more lactic acid present. This would cause a change in taste and smell, rendering it unfit for human consumption. In the dairy industry, pH is an important

quality parameter. In any dairy industry, the quality of raw milk, as well as the finished product, must be monitored and maintained, whether during packaging for human consumption or subsequent processing of other dairy products. Depending on the source of the milk, the pH of fresh raw milk typically ranges from 6.4 to 6.8 Values greater than 6.8 indicate that the milk has coagulated and a value below 6.4 indicates the presence of colostrum or bacterial contamination,

to determine the pH of the milk, electrodes are dipped into the solution and held there long enough for the hydrogen ions in the solution to equilibrate with the ions on the surface of the bulb on the glass electrode. This equilibration ensures a consistent pH measurement (Najim *et al*., 2012).

3.4.2.2 catalase test

The action of two enzymes decomposes hydrogen peroxide: catalase and either a peroxidase, NADH, NADPH, cytochrome c, or glutathione. Catalase reagent, a dilute solution of hydrogen peroxide, is added to a pure bacterial culture to observe the action of these enzymes. Because oxygen is a byproduct of hydrogen peroxide decomposition, any immediate bubbling indicates a positive result, this reagent was prepared in 3% concentration of H2O2.

https://www.dalynn.com/dyn/ck_assets/files/tech/RC35.pdf.

Procedure (Slide Method)

1. Pick the center of a colony derived from an overnight culture plate with a sterile inoculating loop or wooden applicator stick and place it on a clean, glass slide.

2. Apply one drop of catalase reagent to the smear.

3. Look for immediate bubbling. To detect weakly positive reactions, a hand lens may be required

3.4.2.3 Chloride test (Mohr's direct titration)

Normal amount of chloride in milk ranges between 0.097% to 0.14%, chloride readings exceeding 0.14 or 0.16 percent have been reported to indicate aberrant milk brought on by an udder problem, Chloride test One of the standard tests used in the diagnosis of mastitis in dairy cows is the determination of chloride in milk (Najim *et al*., 2012).

Procedure

Suspension of sample in water followed by titration of chloride ion with standardized silver nitrate using potassium chromate as indicator, the chloride combines with silver and the

amount of excess silver is determined by titration, the endpoint color changes from pure white to blood red and is comparatively distinct and easily read.

3.4.2.4 white side test

The amount and opacity of a precipitate which is formed when milk and sodium hydroxide 4% are mixed together, roughly parallel the somatic cell count of the milk, according to (Najim *et al*., 2012).

Procedure

A blank bake sheets on one side, area of about 4 cm in which 5 drops of milk with 2 drops of NaoH mixed together by stick with circular motion and score the reaction using the picture guide.

3.4 Antimicrobial Susceptibility test

Various types of antibiotics were used, where the previously prepared muller Hinton agar was inoculated with a bacteria suspension containing a concentration of 3 x 10^8 cells/cm3 using the plating method by immersing a sterile cotton swab in the suspension and removing the excess from the suspension by pressing the swab and rotating it on the inner wall of the tube and spreading it Then the plates were left to dry for 5 minutes, then the plates were placed using sterile forceps, and then the plates were incubated at 37 °C for 24 hours (Hammer *et al*., 1999), then the growth inhibition area was measured according to the method of the World Health Organization and the use of tablets the following antibiotics:

Table (3-8) Antibiotic used in Susceptibility test

Disk symbol	Antibiotic	Concentrate mg/Disk
C30	Chloramphenicol	30
CIP5	Ciprofloxacin	5
CRO30	Ceftriaxone	30
AMC30	Amoxillin	30
N30	Neomycin	30
CN10	Gentamicin	10

3.5.1 Genomic DNA extraction

The existence of genomic DNA was verified in all synthesized samples using 0.7 percent agarose gels and ethidium bromide staining (Dmitriy *et al*., 2006). By employing the primer sequence listed in the table and a PCR experiment, Listeria monocytogenes isolates were

found (3-4). Samples were repeatedly propagated in the Fraser broth and grown on certain media Oxford (Oxoid). Using PCR, L. monocytogenes was used to identify suspect colonies of Listeria spp. Four primers were chosen based on the genes for the invasive association protein of L. monocytogenes, as shown in the table (3-4). the thermal cycler was used to carry out the DNA amplification procedures. The cycle parameters for PCR shown in table (3-5).

Procedure:

Acccording to Dmitriy *et al*., (2006).

-Harvest the overnight culture cell 1 ml ~2 ml by centrifuge at 13,000 rpm for 30 seconds with 1.5 ml tube.

- Discard the supernatant

-Add 500 µl of Lysozyme Buffer and 20µl of Lysozyme (50mg/ml) and resuspend the cell pellet by pipetting or vortexing.

-Incubate it into 37°C water bath for 60 minutes.

-Centrifuge at 13,000 rpm for 3 minutes and discard the supernatant.

-Add 200 µl of Lysis solution and 20 µl proteinase K solution (20mg/ml) and resuspend the cell pellet by pipetting or vortexing.

- Incubate it into 56 C water bath for 10 minutes.

- Add 200 µl of Binding solution and 200 µl of absolute ethanol and mix well by pulse-vortexing for 15 sec.

- Centrifuge at 13,000 rpm for 3 minutes.

- Carefully transfer 500 ~ 600 µl of supernatant without pellet into the upper reservoir of the spin column with 2.0 ml collection tube without wetting the rim.

- centrifuge at 13,000 rpm for min.

- Add 500 µl of washing 1 solution to the spin column with collection tube and centrifuge at 13,000 rpm for 1 min.

- Add 500 µl of washing 2 solution to the spin column with collection tube and centrifuge at 13,000 rpm for1 min.

- Dry the spin column by additional centrifugation at 13,000 rpm for 1 min to remove the residual ethanol in spin column.

- Transfer the spin column to the new 1.5 ml micro-centrifuge tube (Not provided).

- Add 100-200 µl of Elution solution to the spin column with micro-centrifuge tube, and let stand for at least 1 min.

- Elute the genomic DNA by centrifugation at 13,000 rpm for 1 min.

Table (3-9): Thermal cycling protocols for detection of *L. monocytogenes*.

species	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
L.Monocytogenes	95c 5 minutes	95c 45 seconds	50c 1 minute	72c 1 minute	72c 10 minute

3.5.3 DNA extraction kits

DNA Extraction Kit is designed for rapid isolation of genomic DNA from microorganisms, such as bacteria and parasite…etc, in milk samples.

Table (3_10) DNA extraction kit content

No	Contents	Amount
1	STI Buffer	85 ml
2	ST2 Buffer	30 ml
3	ST3 Buffer	160 ml
4	Wash Buffer2	25 ml
5	Elution Buffer	30 ml
6	Inhibitor removal column	100 pcs
7	GD column	100 pcs
8	Bead beating tube (type c)	100 pcs
9	2 ml centrifuge tube	100 pcs
10	2 ml collection tube	100 pcs

3.5.4 Protocol

1. Sample Lysis

Transferred 200 mg of milk sample to a Bead beating tube containing ceramic beads, then 800 µl of ST1 Buffer was added, the sample were Vortex briefly then incubate at 70 ºC for 5 minutes, the Bead beating Tubes were attached horizontally to a standard vortex by taping or using an adapter, the sample were vortex at maximum speed for 10 minutes at room temperature. The Bead beating Tubes were centrifuged at 8,000 x g for 2 minutes at room temperature to eliminate the foam caused by detergents present in ST1 Buffer, 500 µl of supernatant was transferred to a new 1.5 ml micro centrifuge tube.

2. PCR Inhibitor Removal

Added 150µl of ST2 Buffer, then vortex for 5 seconds, and incubate at 0-4 ºC for 5 minutes, the tubes were centrifuged at 16,000 x g for 3 minutes at room temperature to precipitate insoluble particles and PCR inhibitors. Inhibitor Removal Column (purple ring) was placed in a 2 ml Centrifuge Tube, 500 µl of clear supernatant was transferred to the Inhibitor Removal Column, the tubes centrifuged at 16,000 x g for 1 minute at room temperature then the column was discarded, the flow-through was saved in the 2 ml Centrifuge Tube for DNA Binding.

3. DNA Binding

800 µl of ST3 Buffer was added to the flow-through then mixed by shaking vigorously for 5 seconds, GD Column (green ring) was placed in a 2 ml Collection Tube.,700 µl of sample mixture was transferred to the GD Column, the tubes was centrifuge at 16,000 x g for 1 minute at room temperature then the flow-through was discarded , the GD Column was placed back in the 2 ml Collection Tube, the remaining sample mixture was transferred to the GD Column, the tubes was centrifuged at 16,000 x g for 1 minute at room temperature, the flow-through was discarded then the GD Column was placed back in the 2 ml Collection Tube.

4. Wash

400 µl of ST3 Buffer was added to the GD Column, the tubes were Centrifuged at 16,000 x g for 30 seconds at room temperature, the flow-through was discarded then the GD Column was placed back in the 2 ml collection Tube, 600 µl of wash Buffer was added to the GD Column, the tubes were centrifuged at 16,000 x g for 30 seconds at room temperature, the flow-through was discarded then the GD Column was placed back in the 2 ml Collection tube. 600 µl of Wash Buffer was added to the GD Column again, the tubes were centrifuge at 16,000 x g for 30 seconds at room temperature, the flow-through was discarded then the GD Column was placed back in the 2 ml Collection tube, the tubes were centrifuged at 16,000 x g for 3minutes at room temperature to dry the column matrix.

5. Elution

The dry GD Column was transferred to a new 1.5 ml micro centrifuge tube. 100 µl of preheated Elution Buffer was added into the center of the column matrix, the tubes were let stand for at least 2 minutes to allow Elution Buffer, then the tubes were centrifuged at 16,000 x g for 2 minutes at room temperature to elute the purified DNA.

3.5.5 PCR products analysis (Gel Electrophoresis)

DNA samples were monitored using horizontal agarose gel electrophoresis according to (Bartlett, 2001).

The PCR products was analyzed by agarose gel electrophoresis as the following steps:

1.Prepared 1% Agarose gel by using 1X TBE and dissolving in water bath at 100 C for 15 minutes, after that, left to cool 35 C.

2.Then 5 µl of Ethidium bromide stain were added into agarose gel solution.

3.Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 5 µl of DNA sample were added in to each comb well and 100 bp DNA size marker was loaded along with experimental samples.

4.The gel tray was fixed in electrophoresis chamber and fill by 1XTBE buffer Then electric current was performed at 45 volts (5V/cm between electrodes) for 1hour.

5.PCR products were visualized by using UV Trans illuminator.

3.5.6 Measurement the purity of DNA

Nano drop was used to measure the extracted DNA purity by measuring the absorbance at (260/280 nm) and quantifying the DNA concentration (ng/L) (Adams and Otárola-Castillo, 2013).

The DNA purity examined as following steps:

1-The Nano drop software was opened, and a suitable application was selected (Nucleic acid, dsDNA).

2-A dry wipe was used to clean all device parts, particularly those connected to measurements, and then the system was blanked by carefully pipetting

3-free nuclease water 2 µl on the surface of the lower measurement platforms.

4-After closing the sampling cover and clicking the okay button to start the Nano drop process, 1 µl of DNA was put to the measuring pedestal after it was cleaned.

3.5.7 The Preparation of Primers

All primers used in this study (Table 3-4) were prepared according to the recommendation of the manufactured by dissolving a lyophilized primer in appropriate volume of nuclease free water to yield 100 pmol/ µl as a stock solution A working solution was prepared with the final concentration 10 pmol/ µl by dilution methods.

3.5.8 DNA sequence method

The study of genetic variation between local listeria monocytogenes isolates which was carried out using the DNA sequencing process.

The positive PCR genes products were sent to Macrogen Company in Korea via DHL in an ice bag for DNA sequencing using an AB DNA sequencing device the evolutionary distances were calculated using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method using Molecular Evolutionary Genetics Analysis version 6.0 (Mega 6.0) and Multiple sequence alignment analysis of the partial genes based on Clustal W alignment analysis.

3.5.9 Statistical Analysis

Data were analyzed using SPSS (version 14.0), one-way ANOVA test were used to determine the significance between classes, value $P<0.05$ considered statistically important.

Chapter Four: Results and Analysis

4. Results

4.1 Results of clinical study:

The results of clinical study for 100 cows suspected of being infected with *L. Monocytogenes* in farms, and 150 samples of dairy products (cheese, yogurt, butter) from markets and sell points. No obvious clinical signs have been showed on animals from which samples were taken with recorded previous case history of abortion, 25 sample of milk and dairy products were positive for *listeria monocytogenes*, with 225 negative samples.

13 sample of raw and un pasteurized milk, 4 sample from cheese ,5 sample of yogurt and 3 sample of butter that all these samples made from raw and un pasteurized milk, as mentioned in **table (4-1)** below.

Table (4-1) Number of positive samples of milk and dairy products

Sample	Number	Number of positive samples	Number of negative samples	Percentage
Milk	100	13	87	13 %
Cheese	50	4	46	8 %
Yogurt	50	5	45	10 %
Butter	50	3	47	6 %
Statistical analysis	**X^2=2.11, P>0.05, df=3**			

4.2 Result of growth on culture media

On Oxford agar most *L. monocytogenes* strains formed 1 mm diameter black colonies surrounded by black haloes after 24 h. After 48 h typical colonies were 2-3 mm in diameter, black with a black halo and sunken center **figure (4-1).** Other Listeria spp. had a similar appearance. When examined before 24 h, growth of Listeria spp. was sometimes apparent but without the characteristic blackening. Colonies of *L. monocytogenes* were generally obvious and large on the oxford agar, the aesculin reaction develops after visible colony formation, for this reason, typical appearances may not be seen after overnight incubation. However, it is worthwhile making a preliminary reading of the plates after 24h since only 1% of the positives detected in the present series failed to show visible growth on the first day after inoculation.

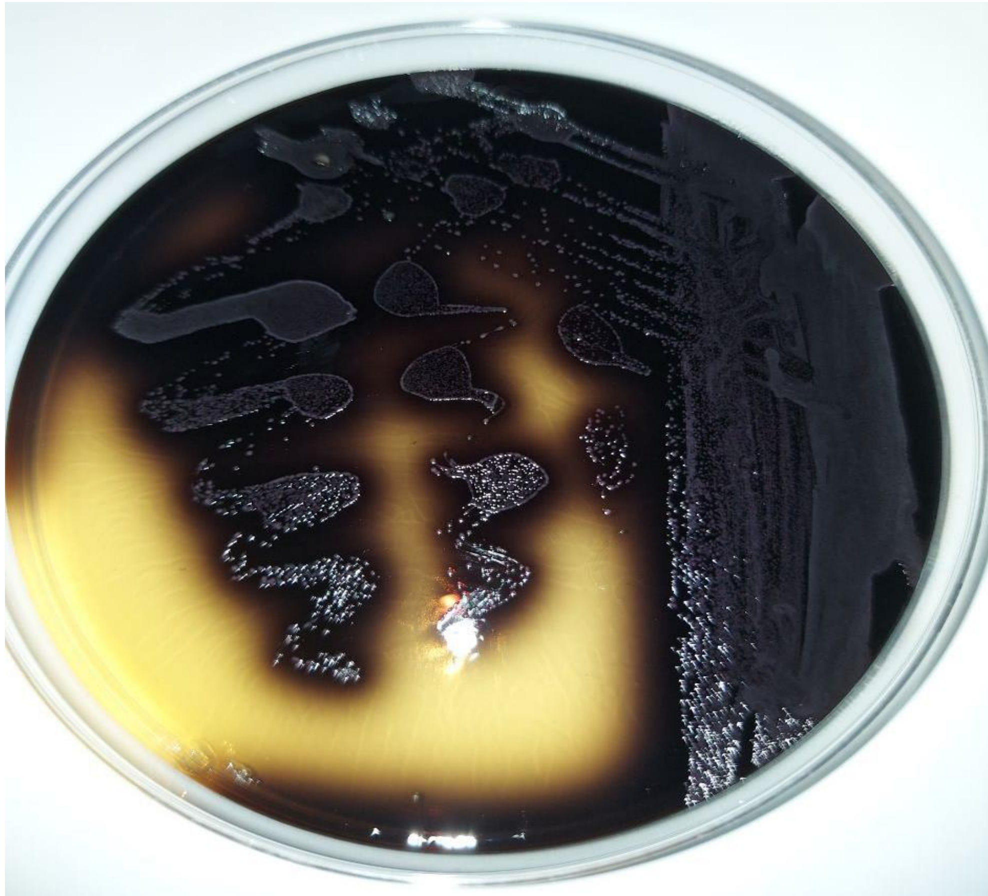


Figure (4-1) Black colonies of *L. monocytogenes* on oxford agar

4.3 Result of microscopic examination

The results of microscopic examination of the prepared and dyed smears showed the characteristic form of the cells of *L. monocytogenes* were Gram-positive with different shapes, including single bacilli, pairs, or in the form of short chains, in addition to some of them giving shapes to different letters, which are T, V, Y and others, as in the **figure (4-2).**

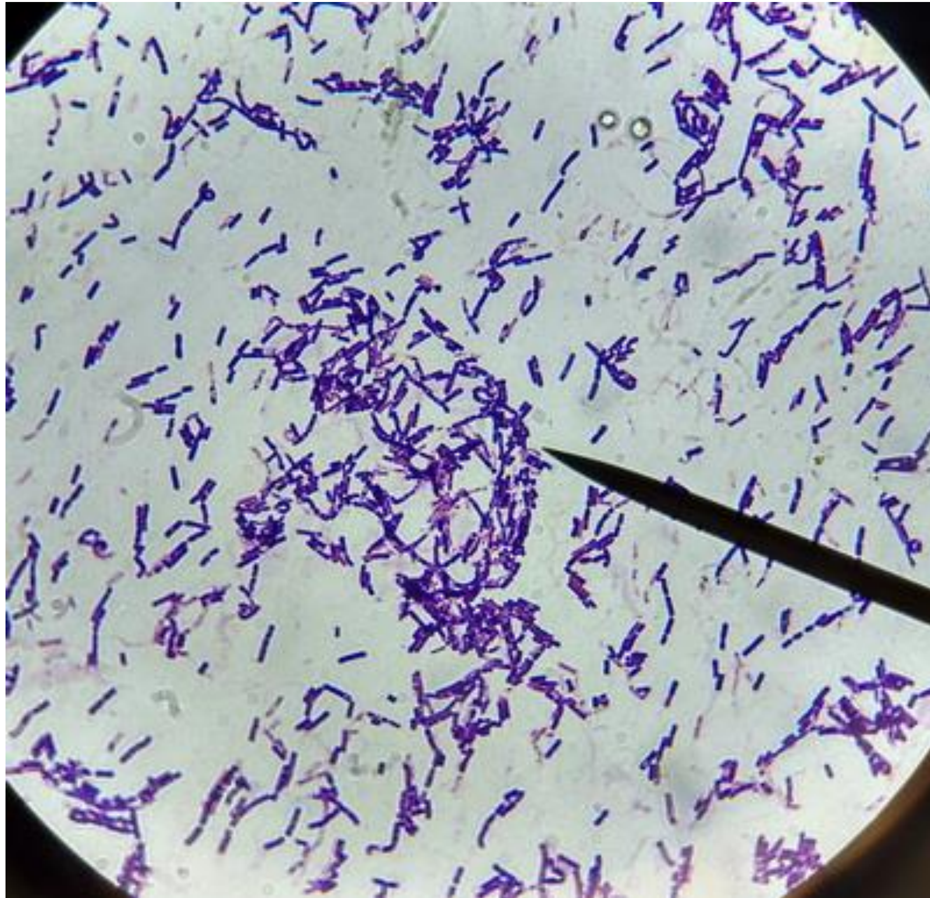


Figure (4-2) ***Listeria monocytogenes*** **Gram Stain as seen in Microscope**

4.4 Results of Molecular Study

Result of Conventional PCR

The result of PCR on 250 samples from cow milk and dairy products have been showed the amplification, which was performed on the DNA, extracted from all the studied isolates confirmed by electrophoresis analysis, By this analysis the strands of DNA which are resulted from the successful binding between specific primer for listeriolysin O gene (hly) and extracted DNA from *listeria monocytogenes* these successful binding appears as single band under the U.V light using ethidium bromide as a specific DNA stain. the electrophoresis also used to estimate DNA weight depending on DNA marker (1500 bp DNA ladder) (Figure 4.4).

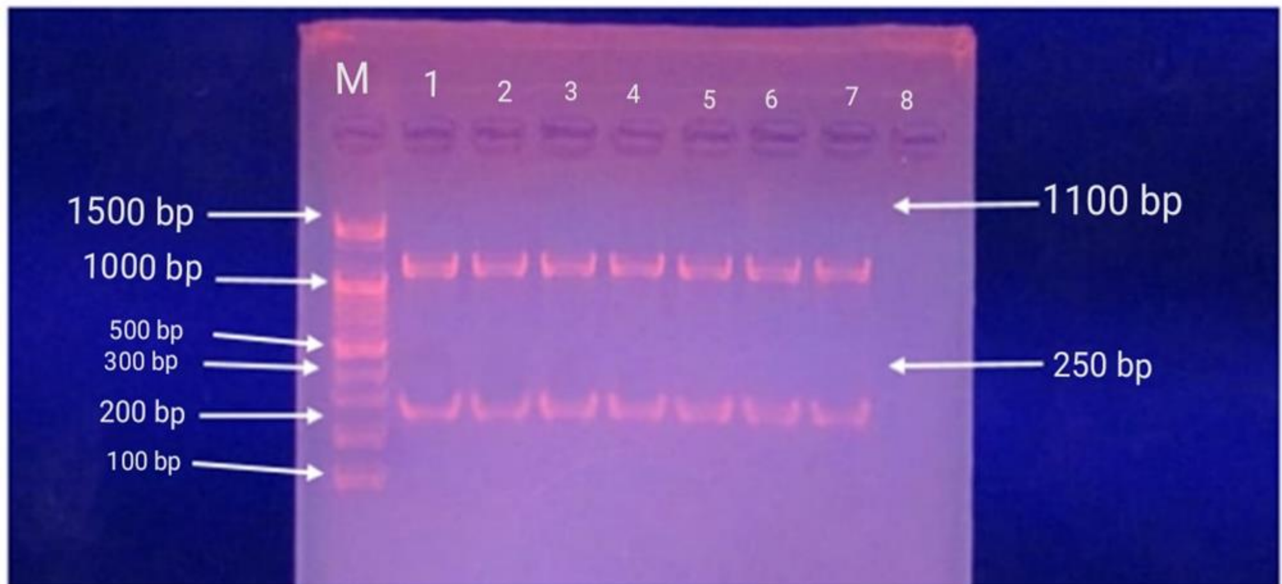


Figure (4-3) Agarose gel electrophoresis of PCR products. M: 1500 bp DNA ladder, lines (1-8) positive result at 250 bp, 1100 bp for Listerolysin O (hly) gene of *Listeria monocytogenes*

4.5 Result of catalase test for *L. Monocytogenes*

This test is used to detect organisms that produce the catalase enzyme. By breaking down hydrogen peroxide into water and oxygen gas, this enzyme detoxifies it. The production of oxygen gas bubbles clearly indicates a catalase positive result as obvious in **fig 4.5**.

Figure (4-4) Catalase test of *Listeria Monocytogenes*

4.6 Result of Antibiotic susceptibility test

The results of this test showed that all isolates showed sensitivity to the antibiotics (Neomycin, Ciprofloxacin, Gentamycin, Chloramphenicol) Whereas, resistance to the antibiotics (Amoxilin, Ceftriaxone) as shown in **table (4- 2)** and **fig (4-5).**

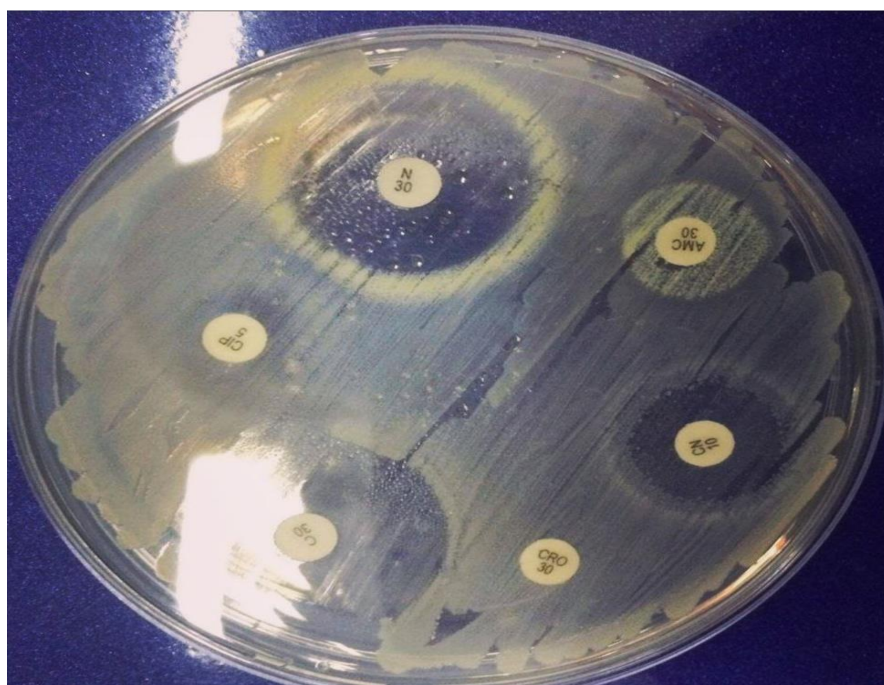


Figure (4-5) Antibiotic Sensitivity test of *L. Monocytogenes*

Agent	Abbreviation	potency	Inhibition Zone diameter (mm)		
			Resistant	Intermediate	Susceptible
Neomycin	N	30	**≤ 22**	**23-27**	**≥ 28**
chloramphenicol	C	30	**≤ 16**	**17-19**	**≥ 20**
Gentamycin	CN	10	**≤ 11**	**12-14**	**≥ 15**
Ciprofloxacin	CIP	5	**≤ 6**	**7-9**	**≥ 10**
Amoxilin	AMC	30	**≤ 7**	**8-9**	**≥ 10**
Ceftriaxone	CRO	30	**≤ 4**	**---**	**≥ 5**

Table (4- 2) Antibiotic Zone sizes

4.7 Percentage of infection of *Listeria Monocytogenes* according to months of study

The results were showed a significant difference in percentage of infection of' *Listeria Monocytogenes* among the months of study in each sample, the highly Percentage of

infection was occurred during winter months specially in January and February but low Percentage of infection was occurred in spring and autumn and there were no cases of *Listeria Monocytogenes* recorded in summer months in all samples as in **table (4-3)**

Table (4-3) Percentage of infection of *Listeria Monocytogenes* according to months of study

Months	Samples type			
	Milk	Cheese	Yogurt	Butter
January	7 (53.8%)	2 (50%)	2 (40%)	1 (33.3%)
February	5 (38.4%)	2 (50%)	1 (20%)	1 (33.3%)
March	1 (7.6%)	0 (0)	1 (20%)	1 (33.3%)
April	0 (0)	0 (0)	1 (%20)	0 (0)
May	0 (0)	0 (0)	0 (0)	0 (0)
June	0 (0)	0 (0)	0 (0)	0 (0)
Total	13	4	5	3

4.8 Results of samples distribution according to regions

samples were collected from different regions in Kerbala include 2(8%) center of the city, and 5(20%) in each of (Al-Husainia, Al-Hindia, Ain altamer) where the highest percentage was recorded in the district of Al_gadwal algarbi, where the number of positive samples was 8 (32%) out of 25 samples positive for *Listeria monocytogenes*,

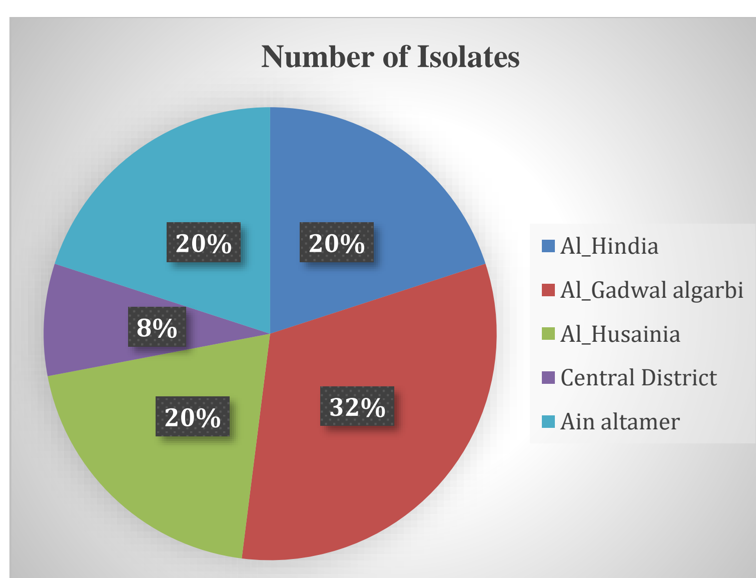


Figure (4-6) Distribution of Isolates by regions

4.9 Results of Physical and Biochemical tests on milk and dairy products samples

4.9.1 Results of Physical tests

Milk samples that suspected of been contaminated with *listeria monocytogenes* showed thick consistency, darker color with yellow tint appear obviously in 50 samples. 25 samples showed unpleasant odor where's, normal milk have Very little distinct odor, milk normally has a slightly sweet flavor, 25 samples showed undesirable salt taste.

All cheese is naturally white, or off white, or even a golden yellow, depending on the type of milk used but abnormal cheese color either darkened or faded in 20 samples ,15 sample show stinky odor, and 15 sample salty flavor suspected with *listeria monocytogenes*, also excess amounts of water have been founded in10 yogurts samples and 22 sample sour small, 18 sample un pleasant taste. different colors of butter vary from black to brown color in 25 sample,5 sample Sour smell, and 20 sample sour bitter taste. these records have been clarified in **table (4_4)** as following:

Type of samples	No of samples	Physical features			Percent%
		color	smell	taste	
Milk	100	Thick consistency, darker color with yellow tint (50)	Un pleasant odor (25)	Un desirable Salt taste (25)	40 %
Cheese	50	darkened or faded color (20)	Stinky odor (15)	salty flavor (15)	20 %
yogurt	50	Excess Amount Of Water (10)	sour smell (22)	sour and unpleasant (18)	20 %

butter	50	Black to reddish brown (25)	Sour smell (5)	sour-bitter taste (20)	20 %
Total	250	105	67	78	

Table (4- 4) physical tests of milk samples and dairy products

4.9.2 Results of Biochemical tests

Physicochemical parameter results of cow raw milk and dairy products samples are shown in **table (4- 5)** In all cases, the health Standards were used as references to milk and dairy products quality recommended levels. of all the samples assessed, it was found that the samples pH was 6.6 ± 0.109 (range 6.3–6.9); the samples that had pH below normal value were 203 (81.2%), but on chloride test, only 29 (11.6%) were below normal. and all the samples tested (*n* = 250) had the levels within the normal range of 4.0–6.8 in all tests, it was also observed that the mean total solids were 14.4 ± 0.4 % and 36 (14.4%) of the samples tested were found to have total solids below normal recommended values.

Table (4- 5) Biochemical results of raw cow milk and dairy products in Karbala.

Physiochemical parameters	Category	Number (%) of milk and dairy products samples from different districts					Total number (%) of milk samples N= 250
		Al-Hindia N= 45	Al-Gadwal Algarbi N= 65	Al Husania N= 55	Ain-Al-Tamer N= 50	Central district N=35	
pH	> 6.8 < 6.4	8 (17.7) 33(73.3)	6 (9.2) 54(83.07)	5 (9.09) 47 (85.4)	6 (12.0) 41(82.0)	6 (17.1) 28 (80.0)	31 (12.4) 203(81.2)
Statistical analysis	X^2= 4.58, P> 0.05, df = 4						
Chloride	> 0.097 < 0.014	4 (8.8) 40(88.8)	10 (15.3) 52 (80.0)	6 (10.9) 47 (85.4)	5 (10.0) 42(84.0)	4(11.4) 30(85.71)	29 (11.6) 211(84.4)

Statistical analysis	**X^2= 2.54, P> 0.05, df = 8**						
White side	< 1 > 3	7 (15.5) 37 (82.2	3 (4.6) 60 (92.3)	17 (30.9) 33 (60.0)	8 (16.0) 41(82.0)	1 (2.8) 33 (94.2)	36 (14.4) 204(81.6)
Statistical analysis	**X^2= 27.52 , P< 0.05, df = 8**						

Chapter Five: Discussion

5. Discussion

5.1 Isolation Ratios

There was no scientific document explaining the registration of *listeria monocytogenes* in Karbala governorate from cow's milk. the results of the study showed that the rate of isolation of *L. monocytogenes* from fresh raw milk and dairy products was 10 % and the high rate of isolation of percentage of *Listeria Monocytogenes* from Al-Gadwal Algarbi (32%) this belong to several reasons, including the lack of preventive measures and health services in the region and large number of animal husbandry rates and their widespread without taking into account preventive measures against diseases, and the lowest percentage in the city center did not exceed 2 samples out of 25 samples positive for *Listeria monocytogenes*, due to the lack of animal breeding in the area and the availability of health services and precautionary measures in the area.

L. monocytogenes can be isolated from raw milk, cheese, and ready-to-eat (RTE) products, most of these items are widely consumed in Iraqi cities. This was found to be a serious public health problem because this bacterium can spread through the consumption of these products, causing different infections, including Human listeriosis (Safana *et al*.,2021). the result show that listeria detection from raw milk in rate of 10 this percentage less than rate that recorded by (AL-Shamary, 2010) which is12.5% in Baghdad that's due to difference in location of study, number of sample and seasonal variation of study. The rate of contamination in this study (10 %) was more than of raw milk contamination levels in other studies in Iraq, as in these studies the rate of contamination was 3.4% in north of Iraq [Erbil] (Alzubaidy,2013). And less than 15.2% in West of Iraq (Noomy,2021) Also, in a study of the Baghdad city (central of Iraq) in 2020, the rate of *L. monocytogenes* contamination 31.1 respectively (Safana ,2021).

In this study, a low prevalence (10%) of *L. monocytogenes* was found in the raw milk samples. Is higher than frequency findings of *L. Monocytogenes* (0-5%) in bulk tank milk samples have been reported from different countries such as Austria 1.5% (Deutz *et al*.,1999) Spain 3.6% (Gaya *et al*., 1998) India 1.7% (Adesiyun *et al*., 1996)

USA 4.1 (Rohrbach *et al*., 1992) Canada 1.9% (Fedio *et al*., 1990) and Iran 1.6% (Moshtaghi *et al*., 2007). The rate of *L. monocytogenes* contamination in raw milk also has been less than 5% in different studies in other countries (Vitas and Garcia ,2004). Winter is best season for listeria dissension, type of diagnosis test predominating PCR is better than culture technique due to their ability to detect low number of bacteria and dead bacteria (AL-Shamary, 2010).

Milk with high rate of water conceder ideal environmental for bacterial growing, also cow milk contain phospholipid which is suitable for listeria (Robinson, 2000). The difference rate of listerial infection depend on type of animal, strain and the age of animal, geographic location (Schlech, 1983). In addition, the recovery of *L. monocytogenes* occurred in all raw milk samples with different variation depending on the concentration of the pathogen inoculums and the levels of microbiota, the recovery of *L. monocytogenes* in raw milk samples may due to the composition of the milk (Robinson *et al.*, 2000). Autochthonous microbiota plays a very important role which represents the main interfering factor such as different types of metabolites produced by microbiota in the milk as an unfavorable condition that can inhibit the bacterial growth or survival by competition (Besse, 2002).

Our values also showed differences when compared to other studies performed in other countries. In Isfahan, Iran, a research was conducted on various food products, including dairy products, meat, and ready-to-eat food, and found a 4.7% contamination rate with *L. monocytogenes* (Jalali M,2008). Which is higher than that found by our study. However, the occurrence of *L. monocytogenes* in the current research was lower than that found in an earlier study (Goh ,2012). Our explanation for the differences between our results and those of the previous works is that a wide range of animal species can become infected with *L. monocytogenes*, including mammals and domesticated animals, research conducted in Iraq found that the highest incidence of listeriosis occurred in the cold seasons (Hussein and Huda ,2015).The disparate levels of contamination which have been reported from localized studies might have been due to variations in regions or to variations in sampling and detection techniques. Therefore, to determine the accurate prevalence of Listeria monocytogenes, further investigations should be carried out

in dairy farms using a large number of samples Because of the presence of calcium ions as a PCR inhibitor in raw milk (Bickley *et al*., 1996).

The persistence of *listeria monocytogenes* cells in yogurt at PH 4.1 was surprising in light of the supposed acid tolerance of the organism (Gray *et al*.,1966). The lowest pH values reported for the persistence of the organism were about 4.6 in cheese (Ryser *et al*.,1987). This study is the first report of *L. monocytogenes* contamination in raw milk used for raw milk and dairy products in Karbala city, However Infected animals and poor silage quality in dairy farms have been considered the source of Listeria spp. in raw milk, as well as environmental contamination which could occur during milking and storage (Sanaa,1993; Sagun,2001).

Therefore, in this study insufficient hygiene and as well as poor silage quality in dairy farms are likely the most common causes of Listeria spp. contamination of dairy products and raw milk with *L. monocytogenes* showed a seasonal trend as the pathogen was first detected in February and then on three more consecutive visits: March, April and June. This timing partly coincides with the consumption of silage by cows in the country during the period they are confined over the winter months (Yoshida *et al*.,1998). Bourry , (1995) has been mentioned that the persistence of infection with sub-clinical mastitis, listeria mastitis, cows not showing any clinical signs go undetected where they may produce milk with normal appearance containing large numbers of pathogenic *L. monocytogenes* (Winter *et al*.,2004).

Clinical mastitis is, by definition, abnormal milk and no reference to SCC is required, the presence of flakes, clots, or other gross alterations in appearance of quarter milk is evidence of clinical mastitis and is by definition, abnormal milk (Kitchen ,1981).

The varied distribution of *L. monocytogenes* serotypes with respect to region may reflect local movement and distribution of animals, feedstuffs, and people (Van Kessel *et al*.,2004).

Listeria monocytogenes is more thermally tolerant than most other non-spore-forming foodborne pathogens. However, current vat (63°C for 30 min) and high temperature– short-time pasteurization (72°C for 15s) practices will ensure total

destruction of *L. monocytogenes*. Despite the ability of *L. monocytogenes* to attain populations of 106 cfu ml_1 in commercial skim milk, whole milk and whipping cream after 8 days of storage at 8 _C (a not uncommon temperature of home refrigerators), this organism has been rarely detected in pasteurized fluid milk products (Ryser.,2007).

Listeria monocytogenes is known to survive refrigeration, dry environments, and the presence of certain inhibitory agents (Doyle *et al*, 2001) It is also able to survive in milk processing plants for up to seven years (Waak *et al*., 2002).

The observed risk factors that are significantly associated with raw milk contamination by *L. monocytogenes* The most important criteria are time sequence, strength of association, biologic gradient, coherence, and consistency and specificity of association, time sequence refers to the fact that the cause precedes contamination, Biologic gradient refers to a relationship between dose and effect, Coherence refers to the biological plausibility given the current state of knowledge, whereas consistency refers to the presence of similar findings in different studies under different conditions, the risk factors presented in the current study should be interpreted via those criteria (Sanaa *et al*.,1993).

The rate of mastitis was associated with the microbiological contamination of the bedding, and dirty cubicles may have increased the exposure of the cow to coliform microorganisms. Apparently, insufficient hygiene in cow housing increases environmental exposure to pathogens, including *L. monocytogenes,* and constitutes an important environmental factor in the incidence of *L. Monocytogenes* in milk (Winter *et al*.,2004). The occurrence of *L. Monocytogenes* in market samples of different food items indicated that 38% of the samples contained *L. Monocytogenes*, of them 49% of vegetables 34% of chickens and 26% of dairy products were contaminated with *L. Monocytogenes.*

In addition, the education status of the farmers and lack of access to dairy extension services by both the farmers and milk collecting centers could have contributed to the high frequency of contamination as reported by previous researchers (El Marnissi *et al*.,2013). The absence of farm entry restriction and biosecurity measures; as encountered in this research has been reported to be a risk

factor for farm environmental contamination with *Listeria monocytogenes* elsewhere Silage has been reported as a major risk factor to Listeriosis; however only 3% of the farms used it to feed the animals; and all milk samples were contaminated with *Listeria*. This is in agreement with what was reported earlier, that ensiling and stored forage is a risk factor for presence of *L. monocytogenes* on farms (David and Cossart., 2017).

To which extent the *L. monocytogenes* develops or survives in milk and dairy products, stored at storage temperature or frozen, depends on the type of dairy product and strain of *L. monocytogenes*, strains of *L. monocytogenes* was isolated from raw milk, cheeses, dairy products and dairy plants (El-Gazar et al., 1992).

According to many authors, *L. monocytogenes* is most commonly isolated from raw milk sampled from collection tanks on farms or in dairy plants, and various contamination degrees have been recorded. In some countries the percentage of contaminated samples was relatively high, which speaks of potential danger from listeriosis if such milk is consumed without prior heat treatment. Presence of *Listeria monocytogenes* in raw milk different from one country to another in Scotland 15.6% prevalence of Listeria monocytogenes have been detected in tanks of farms, 13% and 19.6% in Uganda and Sweden from dairy plants (Waak *et al*., 2002), in raw milk 4% USA (Pearson and Marth, 1990) and 1.17 % Turkey (Tasci *et al*., 2010) ,1.7-3.3 % from raw milk and dairy plants in Iran (Mahmoodi, 2010). In order to curtail the spread of *L. Monocytogenes*, a look into the molecular determinants of its pathogenicity is expedient, undoubtedly, the most characterised virulence gene of the pathogen is the haemolysin gene, hyl (formely called hlya or lisa). This gene codes for listeriolysin O, a pore - forming cytolysin which enables the pathogen to ascape from the phagosome of the host cell other virulent genes in *L. monocytogenes* incloud the plca which cods for a phosphatidylinositol - specific phospholipase ;mpl, which cods for a metalloprotease ; acta required for the polymerization of actin filaments during cell - to-cell spread of the pathogen ;iap gene, responsible for the invasivasiveness of the pathogen ;in la and inlb, coding for internalins and prfa, the virulence regulator (Eneh Chiamaka *et al*., 2019).

Raw milk is one of the most common substrates for the transmission of *L. monocytogenes* as studies have shown that *Listeria* spp. isolates can be usually found in raw milk specimens, studies conducted by Durmaz *et al*. in Turkey showed that the prevalence rates of *L. monocytogenes* in sheep and goat raw milk were 2.7% and 0%, respectively. In another study conducted by Abbas and Jaber (2012). in Iraq, the prevalence of *L. monocytogenes* in sheep milk was 8%. The study conducted by Rahimi *et al* (2018) in Isfahan Province, Iran, demonstrated that the prevalence rates of *Listeria* spp. in sheep and goat raw milk were 22.6% and 6.7%, respectively, and the prevalence rates of *L. monocytogenes* were 6.5% and 1.7%, respectively. In another study conducted by Jamali *et al*. (2019) in Alborz Province, Iran, the prevalence rates of *Listeria* spp. in sheep and goat raw milk were 16.4% and 4.9%, respectively, and the prevalence of *L. monocytogenes* was 4% in sheep and goat raw milk. In another study by Osman *et al*. in Egypt, the prevalence rates of *Listeria* spp. in sheep and goat raw milk were 3.9% and 5.6%, respectively, and the prevalence rates of *L. monocytogenes* were 1% and 1.9%, respectively. Consistent with the findings of Durmaz *et al*. (2015), Rahimi *et al*., and Jamali *et al* (2019) this study showed that the prevalence of *Listeria* spp. was higher in cow milk than in goat milk and there was a significant difference between milk specimens taken from cows and goats in this regard. By contrast, Osman *et al.* in Egypt reported that the prevalence of *Listeria* spp. and *L. monocytogenes* was higher in goat milk than in sheep milk. This study, however, consistent with the results of Rahimi *et al.* (2018) demonstrated that the prevalence of *Listeria* spp. and *L. monocytogenes* was higher in sheep milk than in goat milk in Iran. This can be attributed to the higher health status of the udder in goats than in sheep as well as the higher tendency of sheep to graze and rest in compact groups (K. M. Osman,2022).

5.2 Microscopic examination:

All isolates obtained from animal sources were subjected to studying their phenotypic and microscopic characteristics, and the germ appeared in the form of single or double gram-positive bacilli, and sometimes they appeared in different forms, and this agreed with what was stated in (Baron, 1990) and (Koneman et al., 1997).

5.3 Growth on culture media:

The colonies of L. monocytogenes on Oxford agar were characterized as brown with a black background with the color of the middle changed from yellow to black, this is due to the bacteria's ability to interact esculin with iron ammonium citrate, as well as containing the antibiotics Ceftazidim and Fosfomycin. Which works to inhibit the intestinal microflora, especially enterococci (Curtis *et al*., 1995).

Studies indicate that there are many culturing media for the germ *L. monocytogenes,* most of them contain selective substances such as lithium chloride and acriflavin, as well as antibiotics, esculin and red phenol. The media referred to were selected due to the ease of preparation and availability of their materials in the laboratory, as well as their distinction by rapid investigation of the germ during a period of (24) hours, and this was also confirmed by (Cassiday *et al*., 1989).

5.4 Antibiotic Susceptibility

After conducting antibiotic sensitivity tests, the results of our study were in agreement with many studies which showed that *L. monocytogenes* isolated from different sources, including food and the environment, as well as clinical samples showed sensitivity towards the antibiotic. Bio-Gentamycin, which had the best effect on isolates of this bacterium, followed by the antibiotics Penicillin, then Ciprofloxacin and Chloramphenicol, but our results differed with the researcher Vicente and his group in 1988 who indicated the resistance of the bacterium to the antibiotic Chloramphenicol (Arpin et al., 1992; Charpntier *et al*., 1995). the results of the current study also agreed with what the studies showed that in the resistance of bacteria strains isolated from meat, cheese and ice cream to the antibiotics Tetracyclin and Vancomycin. Also, our results agreed with the results of the researcher Charpentier and his group in 1999 who indicated that the isolated L. monocytogenes from the environment and food, Vancomycin resistance ratios were recorded higher than that of strains isolated from clinical samples, the resistance of the bacteria to Vancomycin antibody was attributed to ribosomal mutations. (Facinelli et al., 1991; Facinelli et al., 1993) As for the resistance of *L. monocytogenes* to the antibiotic Tetracyclin, it may be attributed to the transfer of resistance genes

by conjugative transpons from Enterococcus and Streptococcus bacteria to *L. monocytogenes* as well as their transmission between *L. monocytogenes* isolates, and for this reason some differences appear in the resistance of the strains of the same bacteria, and this was confirmed (Doucet et al., 1991).

Resistance may be attributed to the widespread use of tetracycline as feed additives (Poyart et al., 1992). a resistance to Gentamycin at 4.8%, and this was attributed to the transmission of the resistance gene from Enterococcus fecalis to bacteria *L. monocytogenes* by conjugation method as well as random forage additions, and this was confirmed by researchers (Robert et al., 1996; Charpentier et al., 1995), and our current study agreed with the study of researchers (Charpentier et al., 1999) which showed that isolates of *L. monocytogenes*. monocytogenes isolated from poultry meat recorded resistance to the antibiotic Gentamycin, and this raises many concerns, due to the addition of Gentamycin to the list of antibiotic-resistant bacteria due to acquired resistance. Franco, (1994) have been indicated that the first choice for treatment is Penicillin with Gentamycin, and our study agreed with the researcher's study Laila (2000), which gave its isolates an absolute sensitivity to Gentamycin. while the results of our study differed with the study of the researcher who recorded The strains isolates in her study have an absolute sensitivity of 100% to Chloramphenicol. This may be due to the random use of antibiotics or the relative differences that occur between the different isolates of the bacteria.

Chapter Six: Conclusions and Recommendations

Conclusions

- *L. monocytogenes* was detected in kerbala province with 10 % percentage in raw milk and dairy products (cheese, yogurt, butter).

- The rate of isolation of *L. monocytogenes* from fresh raw milk and dairy products was higher in Al-Gadwal Algarbi than other rates of isolation from other regions with total percentage (32%).

- The infection rates were highly percentage occurred during winter months specially in January and February but low Percentage of infection was occurred in spring and autumn and there were no cases of *Listeria Monocytogenes* recorded in summer months in all samples.

- The bacteria showed absolute sensitivity to Neomycin with sensitivity zone (28 mm), Ciprofloxacin (10 mm), Gentamycin (15 mm), Chloramphenicol (20 mm)] However, resistance to the antibiotics [Amoxilin with resistance zone (10 mm), Ceftriaxone (5mm)].

- From our study the highest infection rates of dairy products founded in yogurt with percentage 10 %.

Recommendations

-In present study confirmed that Hly gene-targeting primers are specific for *Listeria monocytogenes* also showed high incidence of *L. Monocytogenes* in the studied samples.

- Adoption of PCR technique in diagnosing the germ with an attempt to provide several serological profiling kits for the germ for the purpose of accurate profiling of the germ.

- In order to prevent the contamination of milk and dairy products in kerbala province with *L. Monocytogenes* it is necessary to focus and direct the attention on hygiene in
dairy plant production facilities and Sanitation measures.

-By its different characteristics, raw milk products meet the needs of a wide range of consumers. therefore, it’s important to maintain the knowledge about (cheese, yogurt, butter) artisanal production.

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Appendix

Appendix I

1. DNA Sequencing

DNA building blocks (nucleotides) have been determined by whole genome sequencing and decoded, genetic material have been identified by ordering of chemical "letters" of its DNA, each of four letters (A, G, T, C) represents a chemical base, the sequence of the bases can reveal useful information for combatting *Listeria monocytogenes.*

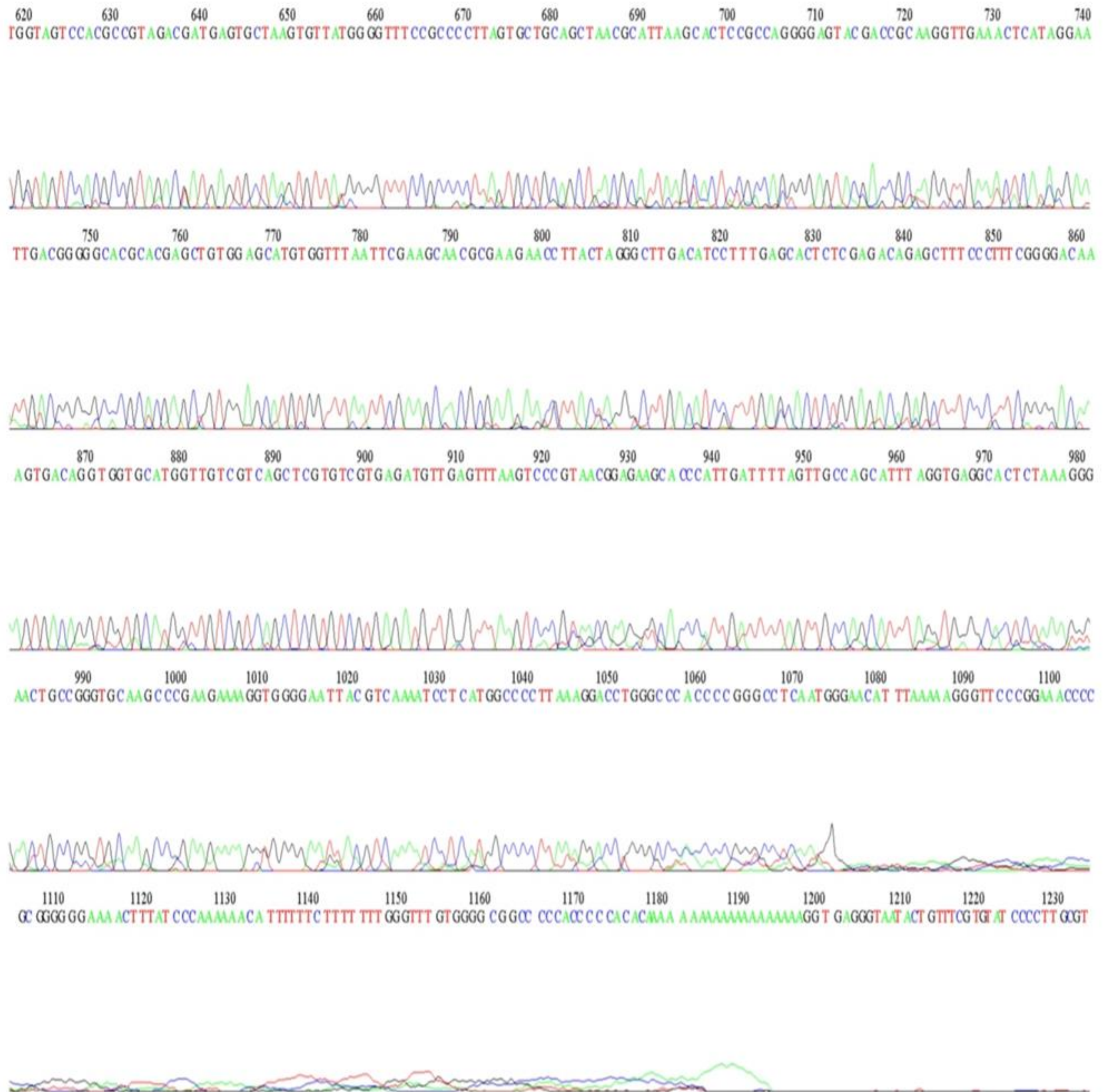

Figure (1) Genomic sequencing in the identification of infectious *Listeria Monocytogenes* variants A, C, G, and T represent letters of the genetic code.

2. PCR-based Methodologies

Conventional Polymerase Chain Reaction (PCR)

Traditional culture-based methods in detection of L. monocytogenes in various environmental settings and food matrices are labor-intensive and time-consuming. PCR-based methodologies have been applied in detection of L. monocytogenes. Conventional PCR is an in vitro enzymatic amplification of a targeted DNA sequence based upon oligonucleotide primer-directed DNA synthesis by a DNA polymerase.

In PCR assay, two primers complementary to 3′ ends of each of sense and anti-sense strand of target DNA are designed to flank the DNA sequence to be amplified. Two primers are supplied in a buffered reaction mixture containing DNA polymerase plus four dNTPs, cofactors (e.g. Mg2+) and DNA template. Its general protocols consist of 3 steps that are temperature-controlled:

(i) denaturation of double stranded DNA template into single stranded DNA at 95oC;

(ii) hybridization (annealing) of two primers to their complementary regions of DNA template at different temperatures set according to melting temperatures (Tm) of two primers for 30-60s; which is typically set at about 3-5oC below Tm of the primers used.

(iii) extension (synthesis) of DNA from the sites dictated by primers at 72°C for 2 min. DNA polymerization proceeds along the region between them. After each cycle, the template number doubles.

As each newly 4 amplified fragment acts as a template in subsequent cycles, successive rounds of temperature cycling can lead to an exponential increase in copy number of target DNA region. A single copy of target DNA is amplified to up to 106 copies in only 30-40 cycles in 2-3 h. PCR amplified products are separated on an agarose gel, stained with ethidium bromide (EtBr), photographed, and the intensities of DNA bands are semi-quantified.

Taq DNA polymerase enables PCR a versatile tool, reducing labor, assay cost, and the potential for error while improving specificity, sensitivity and product yield and lending the assay to automation. Conventional PCR is a relatively easier and less expensive tool to amplify a targeted DNA fragment and useful in detection and monitoring of foodborne microorganisms. Its major disadvantages are relatively labor-intensive and time-consuming with lower detection sensitivity and accuracy, as compared to qPCR mentioned below. It is unable to distinguish viable from dead cells, and gives rise to false-positive results.

Appendix II

Development and Applications of PCR-based Assays in Detection and Subtyping of L. Monocytogenes

Conventional PCR assays for detection of L. monocytogenes in environmental and food samples generally include: enrichment of L. monocytogenes present in the samples by culturing samples in a broth (e.g. Buffered Listeria Enrichment Broth (BLEB) or Brain Heart Infusion (BHI)) with selective agents and subculture to Listeria plating media; DNA extraction; species-specific detection of L. monocytogenes by target gene-based PCR; separation of the PCR-amplified products on a agarose gel; and staining of the separated gel with EtBr, photograph and semi quantitative analysis of amplified DNA bands

Appendix III

Development and Applications of Various PCR-based Assays for Detection of *L. Monocytogenes*

Both conventional and qPCR assays have been applied in detection of L. monocytogenes.

The specificity and accuracy of PCR detection of *L. monocytogenes* depend largely on selection of appropriate target DNA sequences of *L. monocytogenes* to date, the target genes chosen for PCR detection of L. monocytogenes included hlyA gene.

which encodes listeriolysin O (LLO) involved in the lysis of host vacuolar membrane and is present in all L. monocytogenes strains and essential for its full virulence prfA gene, which encodes the central regulator for transcriptional expression of six virulence genes:

iap gene, which encodes p60 with bacteriolytic activity essential for cell viability, haemolysin gene, which encodes a pore-forming hemolysin essential for pathogenicity, inLAB, which encodes internalins A and B related to entry of *L. monocytogenes* into host cells, Lm00733 gene which encodes an aminopeptidase, ssrA gene which codes for tmRNA and functions both as tRNA and mRNA in all bacterial phyla and ImaA, which encodes for an LLO, and *L. monocytogenes* antigen A.

Appendix IV

Serotype- and virulence-specific determination of *L. monocytogenes* with multiplex PCR Assays

A large proportion of human listeriosis cases are caused by infection of *L. monocytogenes* serotypes 1/2a and 4b.

Multiplex PCR Assays have been applied in serotpye- and virulencespecific determination of *L. monocytogenes* isolates. For instance, Liu *et al*. developed a multiplex PCR assay with primers targeting *inlA, inlC* and *inlJ* genes*.* Both 517 bp and 238 bp fragments could be amplified with *inlC* and *inlJ* primer sets, respectively.

The identity of 36 *L. monocytogenes* isolates was confirmed via amplification of an 800 bp fragment with *inlA* primers. The virulence of these isolates was determined by amplification of 517 bp and/or 238 bp fragments. *L. monocytogenes* pathogenic strains able to cause mortality in *A/J* mice via intraperitoneal route could be detected with *inlC* and/or *inlJ* primers. However, naturally nonpathogenic strains were negative with these primers. While 8 of 10 *L. ivanovii* strains were detected by *inlC* primers, they could be excluded as non-*L. Monocytogenes* through their negative reactions with *inlA* primers in multiplex PCR. Thus, application of multiplex PCR targeting *inlA, inlC* and *inlJ* genes allows simultaneous verification of the identity of *L. monocytogenes* species.

another multiplex PCR assay with a combination of five different primers in a single reaction, i.e. one primer was designed based on the conserved 3' end specific for all *Listeria* species and other four primers were specific *for L. monocytogenes, L. innocua,* and *L. grayi*, or three grouped species *L. ivanovii, L. seeligeri,* and *L. welshimeri*, respectively.

The PCR method enabled simultaneous detection of *L. monocytogenes and L.* discriminated multiple serotype and lineage-specific differences among *L. monocytogenes* strains by using a mixed genomic DNA microarray. a PCR-based method for identification of *L. monocytogenes* serotype by designing Division-specific PCR primers from variable regions of *L. monocytogenes.*

17 genomes and using them in conjunction with Division III primer to classify 122 *L. monocytogenes* strains into five serotype groups. The results were consistent with those of conventional slide agglutination method for 97, 100, 94, and 91% of strains belonging to serotypes 1/2a, 1/2b, 1/2c, and 4b.

multiplex PCR assay with primer sets targeting ORF2372 (similar to teichoic acid protein precursor C), InlB (internalin B), inlC (internalin C) and Imo171 (putative peptidoglycan-bound protein).

This assay enabled identification and differentiation of serotypes 1/2a and 4b from other serotypes of *L. monocytogenes.* another multiplex PCR assay by combining detection of *L. monocytogenes* serotypes 1/2a and 4b with ECs I, II, and III with primer sets targeting ECI, ECII

and ECIII This single multiplex PCR allowed determination whether or not an isolate can be genus *Listeria* or *L. monocytogenes* serotype 1/2a or 4b or ECs I, II, III.

A multiplex PCR assay was established with primer sets targeting lmo1118, lmo0737, orf2110, orf2819, *prs* (*Listeria* genus specific), *pfrA* (*L. monocytogenes* specific) and *flaA.*

When being applied to test 187 *L monocytogenes* strains in Japan, 99.5% of them were identified as serotype 4b.

A multiplex PCR with primer sets targeting *prs* (Imo4A_0215, phosphoribosyl pyrophosphate synthetase), *isp* (Im01441, putative peptidoglycan acetylation protein), *L1* (LMOF2365_RS13380, cell wall surface anchor family protein), *L2* (Imo0525, hypothetical protein) and *L3* (LM)4A_RS05595,
hypothetical protein) and applied it to identify genus *Listeria* and discriminate the major lineages among 46 isolates of *L. monocytogenes.*

A multiplex PCR with primer sets specific for serotypes 1/2c (LMOSLCC2372_0308), 3a (LMLG_0742) coupled with primer sets specific for serotype 1/2a (*flaA*) to separate serotypes 1/2a, 1/2c, 3a and 3c, This
assay enabled differentiation of five *L. monocytogenes* subgroups, including 1/2a-3a, 1/2c-3c, 1/2b-3b-7, 4b-4d-4e and 4a-4c, and separation of 1/2a and 1/2c strains from 3a and 3c strains.

By applying two multiplex PCR assays the serotype distribution among 134 *L. monocytogenes* isolates from clinical, beef, and environment samples in Brazil. They found that isolates from clinical samples were mainly serotype 4b while prevalent serotype among beef cut and environment samples was predominantly 1/2c and that results of serotyping with the protocol were completely consistent with those of conventional serology.

Appendix V

Listeria monocytogenes strain AB1 16S ribosomal RNA gene, partial sequence in gen bank

https://www.ncbi.nlm.nih.gov/nuccore/Op218255.1?report=Genbank

الخلاصة

الهدف من هذه الدراسة هو الكشف عن الليستريا مونوسايتوجين في حليب الابقار الخام ومنتجات الألبان حيث تم جمع مائتان وخمسون عينة من الحليب ومشتقاته والتي تشمل (الزبادي ، الجبن ، الزبدة) بشكل عشوائي على فترات منذ بداية كانون الثاني حتى نهاية حزيران ٢٠٢٢ ، في محافظة كربلاء ، تم جمع عينات الحليب من الأبقار في المزارع المحلية بينما العينات من مشتقات الحليب تم جمعها من الأسواق ونقاط البيع ثم تمت معالجة هذه العينات وفقا للمعايير القياسية الخاصه بالليستيريا مونوسايتوجين ونقلها الى المختبر ليتم تحليلها.

أظهرت دراسة الخصائص الفيزيائية لعينات الحليب ومشتقاته الملوثة بـالليستيريا مونوسايتوجين تغيرًا في اللون والرائحة والمذاق بينما اظهرت التفاعلات الكيميائية الحيوية انخفاضا في قيمة الحامضية وزيادة في الكلوريد واعطت تفاعلا ايجابيا في كل من اختبار الكتليز والوايت سايد.

ان الدراسة البكتريولوجية التي شملت عزل وتحديد الليستيريا مونوسايتوجين من مصادر حيوانيه باستخدام اوساط انتقائية خاصة بالليستيريا مونوسايتوجين (اوكسفورد اجار) من شركة هاي ميديا لزرع البكتريا, حيث ظهرت عزلات البكتريا كمستعمرات سوداء واعطت جميعها تفاعل ايجابي لاختبار الكتليز.

تم اجراء الدراسة الجزيئية لليستيريا مونوسايتوجين بواسطة استخدام تقنية PCR والتي تمت من خلال استخلاص الحمض النووي للبكتيريا من خلال استخدام المجموعة الخاصة باستخلاص الحمض النووي.

تم تصميم اربعة برايمرات خاصة ومزدوجة (Hly-1-f, Hly-2-R, List-F, List-R) وفقا للجين الخاص بالليستيريا مونوسايتوجين وهو جينHly. وتم التعرف على عزلات الليستيريا مونوسايتوجين ودراسة التباين الوراثي بين عزلات الليستريا مونوسيتوجين المحلية التي أجريت باستخدام عملية تسلسل الحمض النووي التي تؤكد تلوث عينات حليب الابقار الخام ومنتجات الألبان بالبكتريا.

تمت دراسة حساسية عزلات البكتيريا للمضادات الحيوية, حيث لوحظ أنها حساسة للعديد من المضادات الحيوية تشمل النيومايسين وكان قطر الحساسية 28 مللم, و سيبروفلوكساسين 10مللم وجنتاميسين 15 مللم وكلورامفينيكول 20 مللم وحيث تم تسجيل مقاومة للمضادات الحيوية للاموكسلين وكان قطر المقاومة 10 مللم و السفترازكزون5 مللم.

اختلفت نسبة حدوث الليستريات المستوحدة حسب المناطق من 32٪ في الجدول الغربي إلى 20٪ في كل من والهندية الحسينية وعين التمر، مع نسبة إجمالية قدرها 10٪.

تم الاستنتاج من هذه الدراسة التلوث الكبير في الحليب الخام ومشتقاته (الجبن , الزبادي , الزبدة) بالليستيريا مونوسايتوجين في مدينة كربلاء وضواحيها ويعتبر الحليب ومشتقاته من المصادر الرئيسية

لانتشار ليستيريا مونوسايتوجين الى الانسان.وكانت اكبر نسبة لوجود البكتريا في موسم الشتاء اكثر منه في الصيف خصوصا شهريناير وفبراير.

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كلية الطب البيطري

الكشف عن الليستيريا مونوسايتوجين في الحليب الخام ومشتقاته في محافظة كربلاء

رسالة مقدمة الى مجلس كلية الطب البيطري /جامعة كربلاء وهي جزء من متطلبات نيل درجة الماجستير في فرع الصحة العامة

كتبت بواسطة

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