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Listeria Monocytogenes Presence in Milk and Dairy Products

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The objective of the present study was to detection of Listeria monocytogenes. In raw milk and dairy products. two hundred fifty Samples of milk and dairy products which include (yogurt, cheese, Butter) were collected randomly in interval from January to Mars 2022, in Karbala province, the samples of milk have been collected from cows in local farms while the samples of dairy products were collected from markets and sail points, then the samples were processed according to standard protocols of Listeria monocytogenes and delivered to laboratory for analyzed. the study of physical features for the samples of milk and dairy products which contaminated with L.M have been revealed change in color, smell and taste, while the biochemical reactions have been showed decrease in PH, increase in chloride while catalase and WST were given positive reaction. the bacteriological study which included isolation and identification of listeria monocytogenes from animal sources by using selective media for listeria monocytogenes, oxford agar (hi media) for culturing. The isolates of L.M have revealed as black colonies also all isolates of L.M were given positive results for catalase test. molecular study of L. Monocytogenes was done by conventional PCR technique after DNA extraction using a DNA extraction Kit. four specific multiplex primers (Hyl-1-f, Hyl-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. L.M were reveled highly sensitivity (62.50%) to PCR than culture while the specificity (95.24%) have been showed high in culture than PCR.

Key words: Listeria Monocytogenes, Raw milk, Dairy products contamination.

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Introduction

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).

Materials and Methods

The Animal

The case history of the animal that suspect of infecting with Listeria Monocytogenes was taken, animals with previous abortion cases is preferred for taken samples of raw milk also general examination of the udder and teats and detection of the infected part.

Culture Media

listeria selective agar (oxford formulation) oxford agar is a selective and diagnostic medium 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 and food specimens.

Gram Stain Solution

Gram staining is essential for phenotypic characterization of bacteria; the staining procedure distinguishes Bacteria organisms based on cell wall structure. Gram-positive cells have a thick peptidoglycan layer and appear blue to purple when stained. Gram-negative cells are distinguished by a thin peptidoglycan layer that stains red to pink. Gram used Gentian Violet as the primary stain in the Gram stain at first. Nowadays, crystal violet is commonly used. Hucker's method incorporates ammonium oxalate to prevent dye precipitation (McClelland, 2001) and employs an alcoholic solution of the counterstain. Burke's Gram Stain modification adds sodium bicarbonate to the crystal violet solution. Sodium bicarbonate prevents acidification of the solution as iodine oxidizes (McClelland, 2001), and an aqueous solution of Safranin is used as a counterstain (Gephardt *et al*., 1981).

Identification of listeria monocytogenes by conventional PCR

the polymerase chain reaction (PCR) is a test tube system for DNA replication which allows a “target” DNA sequence to be selectively amplified several million fold in just a few hours. The PCR achieves amplification of a predetermined fragment of DNA, a basic PCR set up requires several components and reagents. These components include: https://www.clinisciences.com/en/buy/cat-conventional-pcr-3473.html

•DNA template that contains the DNA region (target) to amplify

•Taq polymerase, a DNA polymerase that is heat resistant, so that it can remain intact during the DNA denaturation process.

•Two primers that are complementary to the 3' ends of each of the sense and anti-sense strand of the DNA target (DNA polymerase can only bind and elongate from a double-stranded region of DNA, and without primers there is no double-stranded initiation site for polymerase to bind).

•Deoxynucleoside triphosphates (dNTPs, sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand.

•Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase

•Bivalent cations, magnesium or manganese ions; generally, Mg2+ is used, but Mn2+ can be used for PCR-mediated DNA mutagenesis, as higher Mn2+ concentration increases the error rate during DNA synthesis

•Monovalent cation potassium ions

DNA Extraction Procedure:

-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.

Results

Results of Clinical Study:

The results of clinical study for 150 suspected cows in farms Livestock fields and 100 samples of milk and dairy products from sail points and markets infected with *L. Monocytogenes* which used for collecting milk and dairy products (cheese, yogurt, butter) 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 below:

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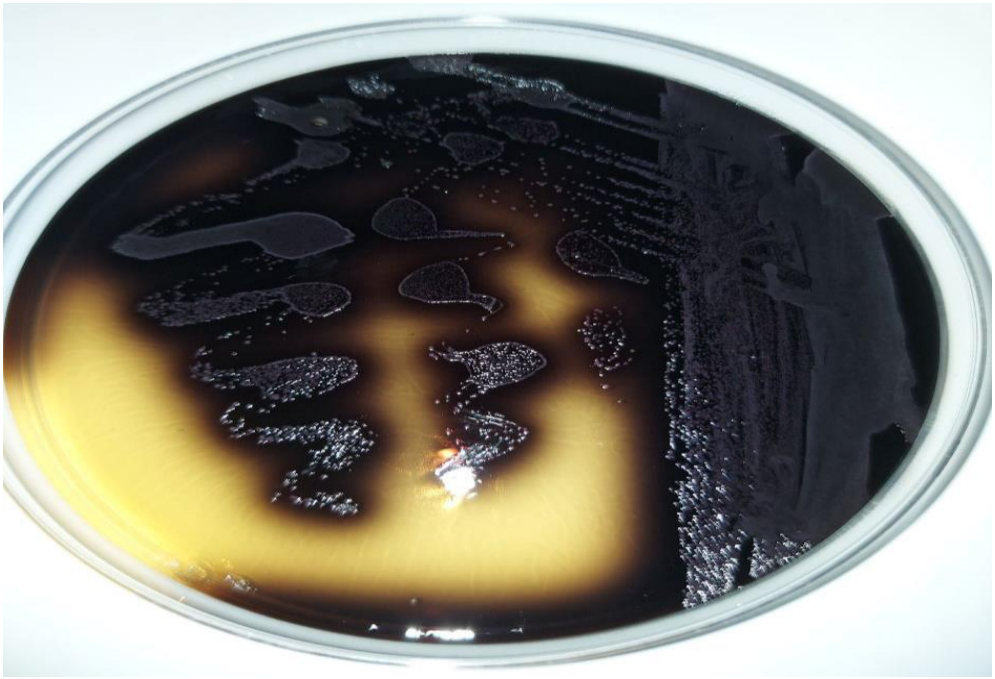
Sample	amount	Number of positive milk samples	Number of negative milk samples
Milk	100	13	87
Cheese	50	4	46
Yogurt	50	5	45
Butter	50	3	47
Statistical analysis	**X^2=2.11, P>0.05, DF=3**		

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 (4-3).** 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. The results of the culturing method of milk samples and dairy products showed that out of 250 samples, the total number of bacterial isolates of *L. monocytogenes* was 25 with a percentage of 10 % which mentioned previously.



Black colonies of *L. monocytogenes* on oxford agar

Result of DNA Sequencing

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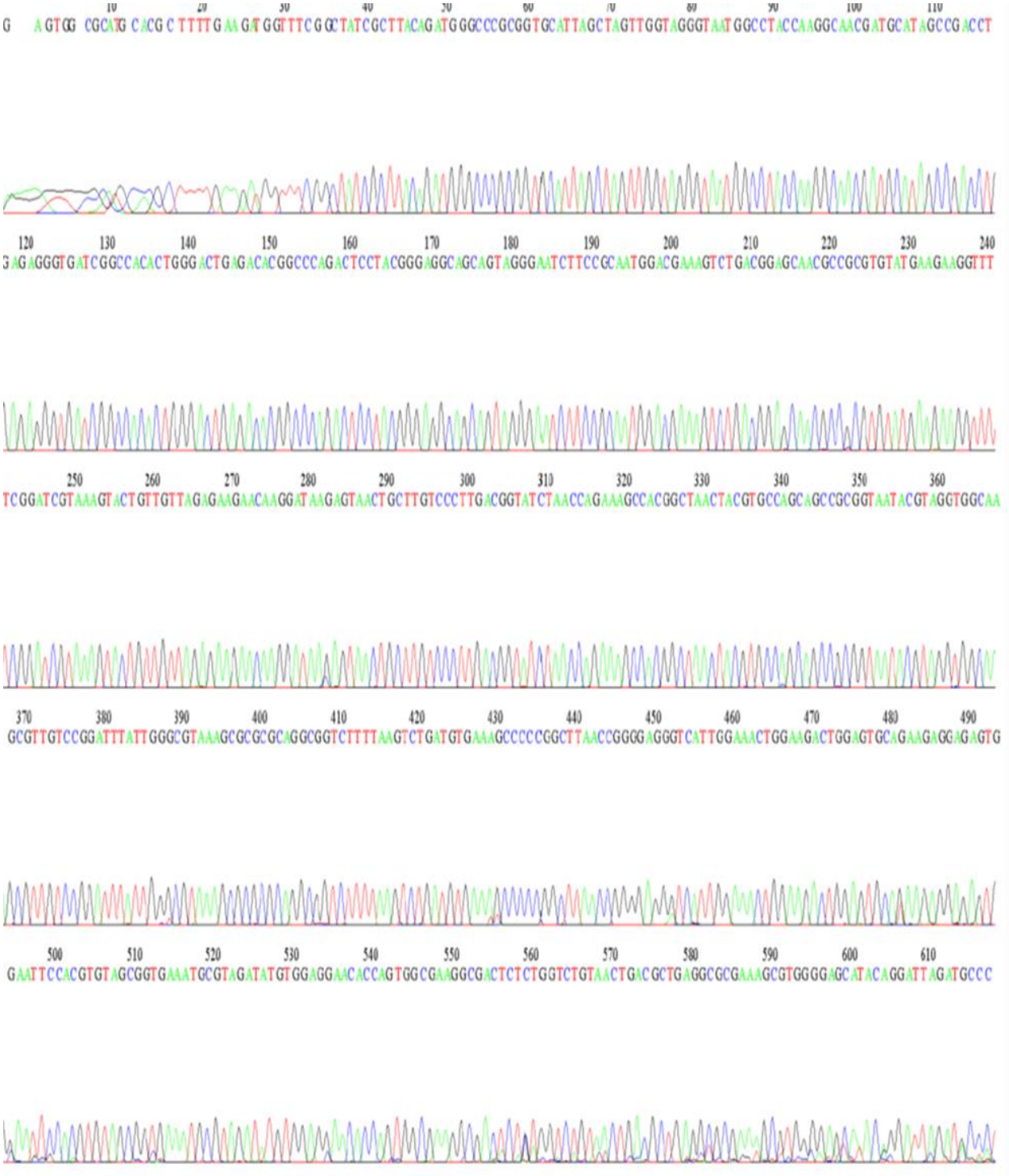
DNA building blocks (nucleotides) have been determined by whole genome sequencing and decoded, genetic material have been identified by ordering of chemical "letters" of its DNA, each of four letters (A, G, T, C) represents a chemical base, the sequence of the bases can reveal useful information for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



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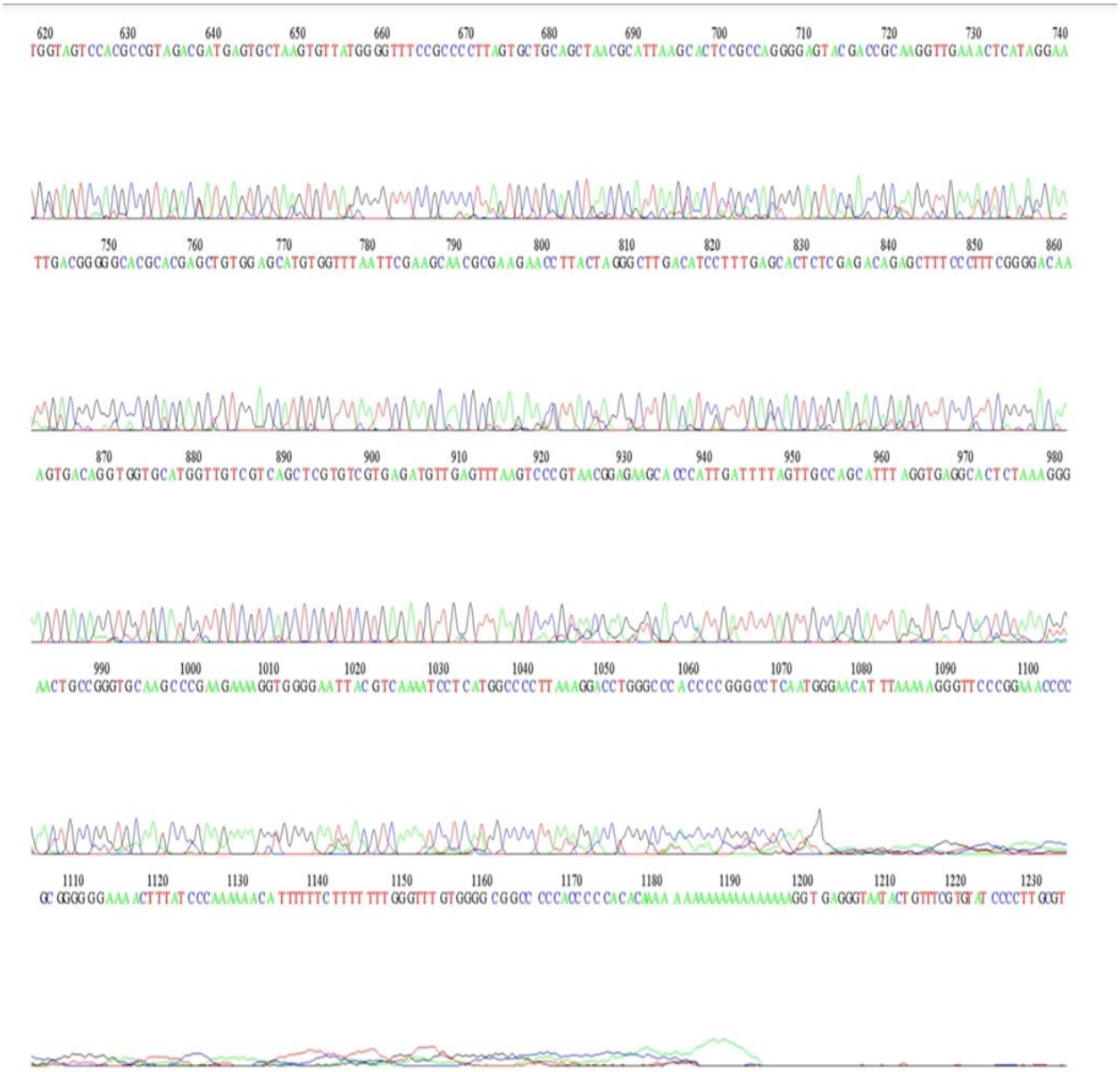

Genomic sequencing in the identification of infectious *Listeria Monocytogenes* variants A, C, G, and T represent letters of the genetic code.

Discussion

Isolation Ratios:

There was no scientific document explaining the registration of *listeria monocytogenes* in Iraq (Karbala) governorate in 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 germs from sail points samples compared to isolation ratio of samples from farms, livestock fields and supermarkets in our study due to Exposing milk samples to the open air, which contributes to providing suitable growth opportunities for the bacteria, in addition to the lack of refrigerated transportation containers means to transport milk from farms and fields to local markets and sail points (Okutani, 2004). *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 (Yassin 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-Shimmery, 2001) 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 in frozen meat of 31.1 respectively (Safana ,2020). 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 ,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-Shimmery, 2001).

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, 1996). 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 ,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.,1996). 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.

We Conclude that Milk and its derivatives have a role in transmitting *L. monocytogenes* to humans and causing disease states.