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RESEARCH ARTICLE

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF LISTERIA SPECIES ISOLATED FROM CHICKEN AND MILK PRODUCTS.

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Abstract

L.monocytogenes is wide spread in the environment and can be isolated from a wide variety of food. As such it can be expected to be present in ready-to-eat foods that contain raw ingredients. The risk caused by *L. monocytogenes* is dependent on the food and how long it is stored. To estimate the incidence and levels of Listeria spp. in different food sources, A total of 200 random samples which collected from different sources 65 samples isolated from poultry , 45 from poultry byproducts (Frozen chicken meat balls (kofta), Frozen chicken burger and frozen chicken sausages), 20 from pasteurized milk ,30 from milk products (Feta cheese ,cream cheese)and 40 from poultry eggs .Only 7(14%) of samples that collected from suspected isolates and detected on Oxford agar, PALCAM agar and ALOA agar were suspected to be Listeria spp. The in-vitro antimicrobial sensitivity test showed that the isolated *L.monocytogenes* were sensitive to Sulphamethoxazole /trimethoprim, Gentamycin ,Ceftazidime and Cephazoline followed by Sulphamethoxazole, ceftriaxone , weak sensitivity to Penicillin , While they were resistant to Erythromycin, Clindamycin, Ampicillin. All *L.monocytogenes* were virulent strains as all of them were positive to CAMP test; showed narrow zone of β–hemolysis on sheep blood agar .The PCR results for Listeria isolates showed that all strains are L.monocytogenes and detect 16SrRNA gene and have *mefA* ,*tet M* and ampC genes (100%) as resistance genes while Cat and Aad6 genes detected as 85.7% and 71.4% respectively.

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Introduction:-

Listeria monocytogenes is one of the sub-species from the genus Listeria. L. monocytogenes is a global concern and can be transmitted by direct contact and digestion and cause a serious diseases: meningitis, abortion, Spontaneous Bacterial Peritonitis (SBP), listeriosis and even death (How et al., 2015).*Listeria monocytogenes* is a Gram-positive rod-shaped non-capsulated bacterium that form single short chains (Theivagt et al., 2006),facultative anaerobic, non-spore-forming, rod-shaped bacteria 0.5 mm in width and 1–1.5 mm in length (Vera et al., 2013).

The organism can survive at varying temperatures ranging from 4 to 37C (Janakiraman, 2008**).** The bacterium can tolerate a wide range of pH and temperatures. Optimum growth occurs at 30-37C but the organism can multiply at4-

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45C. A typical tumbling motility is observed around25C. It can grow at pH 4.5-9.6, although the growth is minimal at low pH and low temperatures (OIE, 2014).

The centralized production of prepared ready-to-eat food products increases the risk of higher levels of contamination, since it requires that foods be stored for long periods at refrigerated temperatures that favor the growth of Listeria. During the preparation, transportation and storage of prepared foods, the organism can multiply to reach a threshold needed to cause infection (Bortolussi, 2008). Egg and egg products have never been caused listeriosis but is most frequently isolated from egg shells and in the environment of laying hens.(Chemaly et al., 2008) *L. monocytogenes* can Survive 90 days on stored egg at 5C and for 15 days at 10C (Gandhi and chikindas, 2007).

Invasiveness by virulence factors, *L.monocytogenes* expresses cell-surface and secreted proteins that enable attachment to host cells, escape from the phagocytic vacuole by Internalin A (inIA) and Internalin B (inIB) mediate the attachment of *L. monocytogenes* to the surface of host cells. Once ingested the bacterium produces listeriolysin (LLO) to escape from the phagosome and lyses the phagosomal membrane,The bacterium then multiply rapidly in the cytoplasm and moves through the cytoplasm to invade adjacent cells by polymerizing actin to form long tails ,the actin-assembly-inducing protein (ActA) which propels bacteria through the cell and into neighbouring cells, (Todar ,2008). *L. monocytogenes* lacking a functional ActA is severely dampened in its pathogenicity. This reduction might not only be due to a lack in intracellular motility however, ActA involved in aggregation and biofilm formation , possibly affecting intestinal adherence.(Travier et al., 2013).

Each step requires expression of specific virulence factors. The major virulence genes are clustered together on the chromosomes and regulated by the positive regulatory factor A protein PrfA (Positive Regulatory Factor A (PrfA). (Scortti et al.,2007 and Freitag et al.,2009).
The present study was conducted to estimate the prevalence of listeria species in poultry and milk products with special interest to *L.monocytogenes*. In addition to carry out the antibiotics sensitivity testing of them and detection of some resistance factors of *L.monocytogenes* by PCR technique.

Material And Methods:-

Samples collection:-
Two hundred random samples which collected from different sources 65samples isolated from poultry , 45 from poultry byproducts (Frozen chicken meat balls (kofta),Frozen chicken burger and frozen chicken sausages),20 from pasteurized milk ,30 from milk products (Feta cheese ,cream cheese) and 40 from poultry eggs. The samples were collected in sterile plastic bags, kept in ice box and transferred with a minimum delay to the laboratory for studying the presence of listeria species.

Bacteriological examination:-
A-Primary stage: One ml of sample was inocluted into 9 ml fraser broth 1,half fraser broth (without supplement) and incubated aerobically at 30±1c for 24±3 hours .B-Secondary stage : one ml of incubated broth was inoculated into 9ml fraser broth 2 , full strength fraser broth (with supplement) and incubated at 37 C for 48±3 hours .C-Third stage :0.1ml of incubated fraser broth was streaked onto the following media : ALOA agar; PALCAM agar and Oxford agar plates then the plates were incubated at 37±1Cfor 48 hours and examined after 24±3 hours. The listeria like colonies were picked and streaked onto Tryptic Soy agar (Bio-life) with 0.6% yeast extract (TSA,YA) then, incubated at 35C for 48 hours. The isolates were morphologically identified by Gram stain and biochemical tests according to (Markey et al.,2013)

In –Vitro anti-microbial sensitivity tests:-
The isolated *L.monocytogenes* strains were subjected to the sensitivity test against different antibiotics, using the disc and agar diffusion method: antibiotic discs are Erythromycin(E), Penicillin (P), Sulphamethoxazole (SM), ceftriaxone (CRO), Gentamycin (CN), Clindamycin (DA), Ampicillin (AM), Cefazoline (KZ), Sulphamethoxazole/ trimethoprime (SXT), ceftazidime (CAZ). (Finegold and Martin, 1982 and NCClS, 1999)

Virulence tests:-
Hemolytic activity: all isolates were cultured on 5% sheep blood agar to determine their hemolytic activity . Also, they were subjected to CAMP test (Mckellar,1994) by streaking *staphylococcus aureus* strains in single straight

lines in parallel on sheep blood agar plates , the isolated listeria strains streaks perpendicularly,(1-2mm).then incubated for 24-48hours at 35C ,enhanced zone of β-hemolysis considered as a positive reaction.

Genotypic detection of isolated L.monocytogenes and some resistance genes in them using polymerase chain reaction (PCR):-

PCR using six sets of primers was used for genotypic detection of *L.monocytogenes* strains by 16SRNA and five resistance genes as macrolides (*mefA*); tetracycline (tetM); β-lactams (ampC) ;chloramphenicol(cat) and aminoglycosides (Aad6).

It was applied on seven isolated *L.monocytogenes* following QIAamp® DNA Mini Kit instructions (Catalogue no. 51304),Emerald Amp GT PCR master mix (Takara) Code No. RR310A and Agarose 1% (Sambrook et al., 1989).The PCR condition can amplify specific products as shown in Table (2). Temperature and time condition of the primers during PCR are shown in table (3) according to specific authors and Emerald Amp GT PCR master mix (Takara) kit.

Results:-

Of the total 200 different samples analyzed, *L. monocytogenes* was detected in 7 samples with prevalence of 14% ; represented as in poultry, poultry byproducts, pasteurized milk, milk byproducts and poultry eggs, listeria percentage were 0/65(0%), 4/45(8.9), 0/20(0%), 0/30(0%) ,3/40(7.5%) isolates, respectively as Table (1) The isolated colonies grow well on ALOA agar producing blue-green colonies surrounded by opaque halo and on PALCAM agar gives grey-green with black center and black halo against cherry-red background and on Oxford agar gives grayish colonies surrounded by black halos. They were Gram – positive bacilli or coccobacilli ;motile showing Umbrella –shaped motility. On biochemical reactions ,they produce acid from L-rhamnose, dextrose and but not with and mannitol. The results of virulence tests showed that , all isolated *L.monocytogenes* strains showed narrow zone of β-hemolysis on 5% sheep blood agar, positive in CAMP test and showed as arrow-shaped zone of weak enhanced hemolysis at junction of tested strains and *S.aureus* strain.

The result of in -vitro sensitivity test phenotypically showed that ,the isolated *L.monocytogenes* were sensitive to Sulphamethoxazole / trimethoprim (100%), followed by Gentamycin, Ceftazidime and Cephazoline (71.4%), followed by Sulphamethoxazole, ceftriaxone (42.8%), weak sensitivity to Penicillin (14.2%). While the isolated were resistant to Erythromycin, Ampicillin and Clindamycin as Fig (1). Agarose gel electrophoresis results of the isolated samples by PCR detected that all the seven isolates were *L.monocytogenes* duo to detection of 16SrRNA gene and genotypic detection of antibiotic resistance showed that all isolates were 100% resistant to β-lactams ,tetracycline and macrolides detected by Ampc, tetM and mefA genes respectively ,while 85.7% of isolates were resistant to chloramphenicol detected by Cat gene and 71.4% of isolates were resistant to aminoglycosides detected by Aad6 gene .

Table 1:-Total number and percentage of positive samples for listeria isolation from examined samples.

			Positive percentage		
Samples	Number of samples	Number of positive samples	%1	%2	%3
Poultry	65	zero	zero	zero	zero
Frozen wings	35	zero	zero	zero	zero
Frozen breast	30	zero	zero	zero	zero
Poultry products	45	4	8.9	57.1	2.0
Frozen chicken meat balls (kofta)	10	2	4.4	28.5	1.0
Frozen chicken burger	15	1	2.2	14.2	0.5
frozen chicken sausages	20	1	2.2	14.2	0.5
Pasteurized Milk	20	zero	zero	zero	zero
Milk products	30	zero	zero	zero	zero
Feta cheese	10	zero	zero	zero	zero
Cream cheese	20	zero	zero	zero	zero
Poultry eggs	40	3	7.5	42.8	2.5
Total	200	7	3.5	100.0	3.5

1. Percentage in relation to total number of samples in each raw.
2. Percentage in relation to total number of positive samples (7).
3. Percentage in relation to total number of collected samples 200).

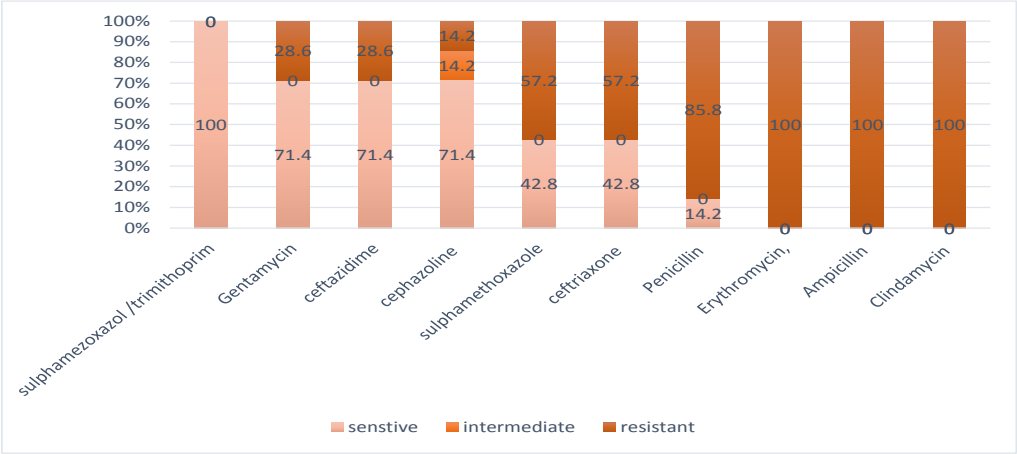

Fig.1:- In-vitro antimicrobial sensitivity test for isolated *L.monocytogenes* .

Table 2:- Oligonucleotide primers sequences sources

Primer	Sequence	Amplified product	Reference
16S rRNA	ggA CCg ggg CTA ATA CCg AAT gAT AA	1200 bp	Kumar et al., 2015
	TTC ATg TAg gCg AgT TgC AgC CTA		
Aad6	AGAAGATGTAATAATATAG	978 bp	Morvan *et al.*, 2010
	CTGTAATCACTGTTCCCGCCT		
Cat	GAACAGGAATTAATAGTGAG	384 bp	
	GGTAACCATCACATAC		
mefA	AGTATCATTAATCACTAGTGC	345 bp	
	TTCTTCTGGTACTAAAAGTGG		
tetM	GTGGACAAAGGTACAACGAG	405 bp	
	CGGTAAAGTTCGTCACACAC		
ampC	TTCTATCAAMACTGGCARCC	550 bp	SRINIVASAN *et al.* 2005
	CCYTTTTATGTACCCAYGA		

Table 3:- cycling conditions of the different primers during cPCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
16S rRNA	94˚C 5 min.	94˚C 30 sec.	60˚C 1 min.	72˚C 1 min.	35	72˚C 12 min.
Aad6	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 50 sec.	35	72˚C 10 min.
Cat	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.
mefA	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.
tetM	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	35	72˚C 10 min.
ampC	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

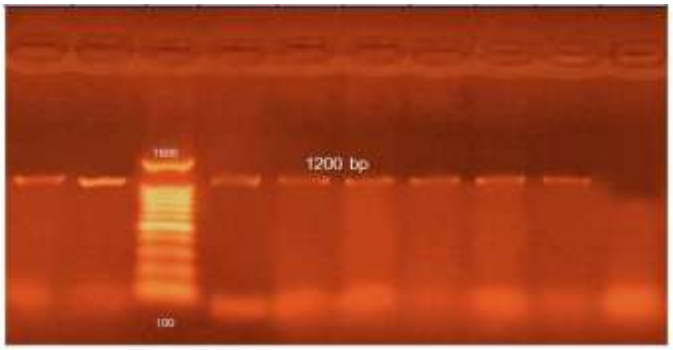

Fig. 2:- 16SrRNA genes . Lane L:100-1500bp Ladder. Neg:Negative control . Pos. :positive control at (1200bp) .Lanes 1 to 7 :*L.monocytogenes* (16SrRNA) gene positive.

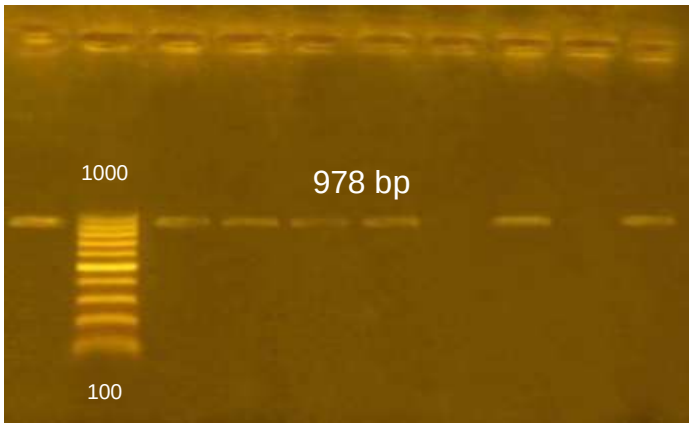

Fig.3:- Aad6 gene. Lane L:100-1000bp Ladder. Neg:Negative control . Pos:Positive control at (978bp). Lanes 1 to 7 :*L.monocytogenes* (Aad6)gene positive except two samples.

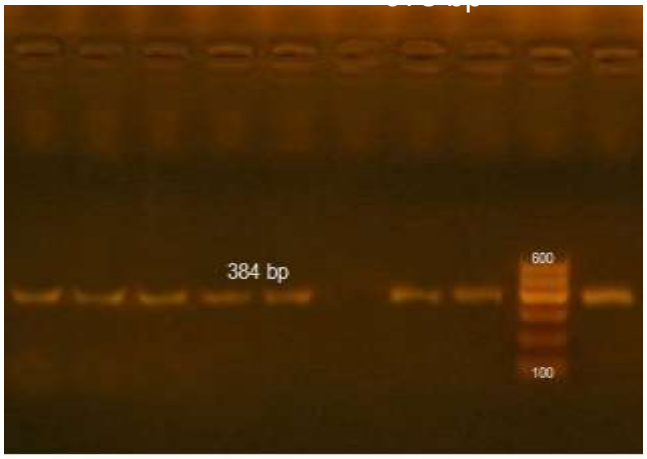

Fig.4:- *Cat* gene. Lane L:100-600bp Ladder. Neg: Negative control . Pos:Positive control at (384bp). Lanes 1 to 7 :*L.monocytogenes* (*Cat* gene) positive in all samples except one sample.

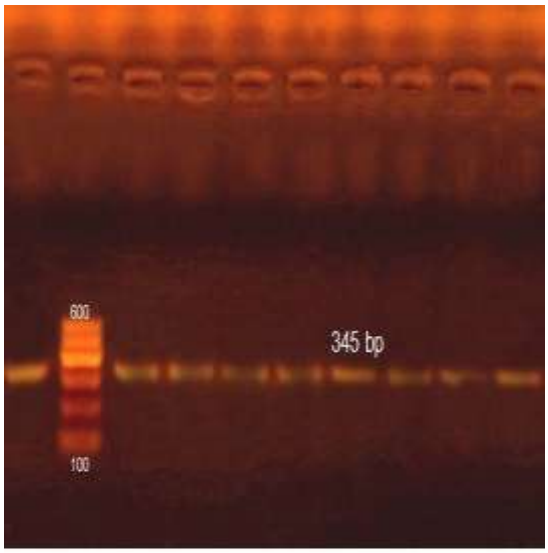

Fig. 5:- Mef*A*gene. Lane L:100-600bp Ladder. Neg: Negative control . Pos:Positive control at (345bp). Lanes 1 to7 *L.monocytogenes* (Mef*A*gene) positive .

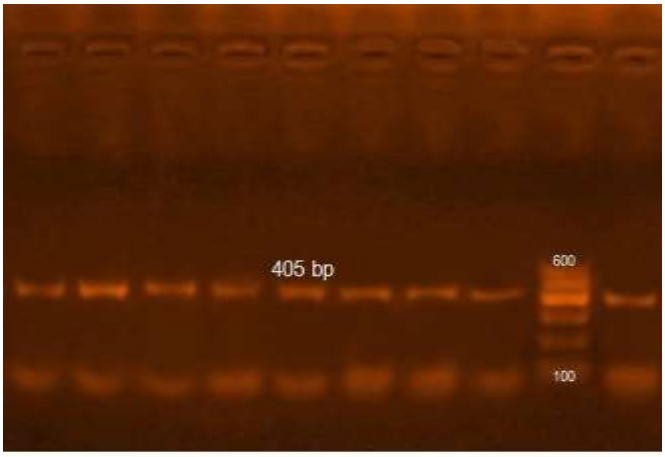

Fig.6:-*tet* M gene. Lane L:100-600bp Ladder. Neg: Negative control . Pos:Positive control at (405bp). Lanes 1 to 7 :*L.monocytogenes* (*tet* M gene) positive .

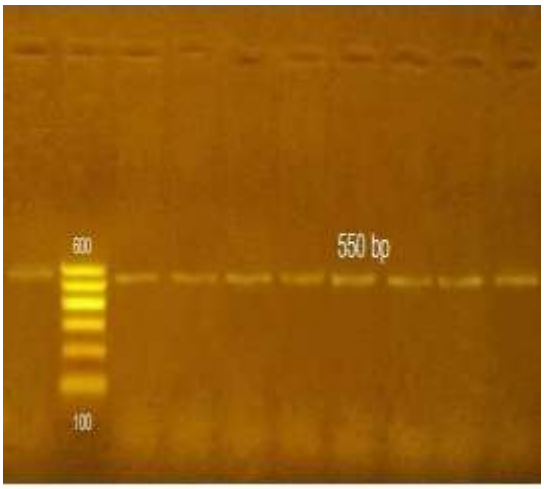

Fig.7:- *AmpC*gene. Lane L:100-600bp Ladder. Neg: Negative control . Pos:Positive control at (550bp). Lanes 1 to 7 :*L.monocytogenes* (*AmpC*gene) positive

Discussion:-

Listeriosis is of great public health concern because of its high mortality (20 to 30%) and its common source epidemic potential. The most important aspect in food hygiene is the ability of the bacteria to survive in a wide range of temperatures and to make biofilms on various environmental surfaces, which serve as natural habitats or reservoirs (Duggan and Phillips, 1998).

Ability of *L. monocytogenes* to cause disease depends upon the expression of virulence factors and immune status of individuals. Usually individuals having weakened cell-mediated immunity are more susceptible to *L. monocytogenes*. (Lecuit et al., 2004)

In this research we found that *L. monocytogenes* was detected in 7 samples out of 200 (14%)represented as in poultry, poultry byproducts, pasteurized milk, milk byproducts and poultry eggs,with percentages 0/65(0%), 4/45(8.9%), 0/20(0%), 0/30(0%) ,3/40(7.5%) respectively that disagreed with that recorded by (Zeinali et al., 2017)

Listeria monocytogenes wasn't found in Frozen wings of poultry, frozen breast of poultry that totally disagreed with (Reiter et al., 2005)

The result of in vitro sensitivity test showed that ,the isolated *L.monocytogenes* were highly sensitive to Sulphamethoxazole /trimethoprim (100%), so we can use it in its medication (Swaminathan and Gerner- Smidt, 2007),followed by Gentamycin Ceftazidime and Cephazoline (71.4%), followed by Sulphamethoxazole, ceftriaxone (42.8%), weak sensitivity to Penicillin (14.2%), while the isolated strains were resistant to Erythromycin, Ampicillin and Clindamycin and these results came in accordance with those recorded by (Zeinali et al., 2017) and disagreed with (Altuntas et al.,2012). All L.monocytogenes isolates were positive to CAMP test and showed narrow zone of β-heamolysis in sheep agar, the similar results were reported by (Marrouf et al ., 2007). Regarding to the occurance of 16SrRNA genes in *L.monocytogenes* isolates , the obtained result revealed that , it was amplified in all seven tested isolates ,that agreed with those recorded by (Ciolacu et al .,2015)

Chloramphenicol encoded by (Cat)gene was amplified in six tested isolates as in Fig.(4),that similar recorded by (Poyart-Salmeron et al.,1990). macrolides encoded by (mefA) gene was amplified in all seven tested isolates Fig(5) that resemble the result of our phenotypic antibiotic sensitivity test Fig(1), and disagreed with (Leclercq,2002).Tetracycline encoded by (tetM) gene was detected in all *L.monocytogenes* isolates that came in accordance with (Granier et al.,2011), 71.4% of the *L. monocytogenes* isolates were resistant to aminoglycosides that encoded by (Aad6) gene that the same reported by (Charpentier et al., 1995) while 100% of the *L. monocytogenes* isolates were resistant to β-lactam that encoded by (Ampc) gene that resemble our result of phenotypic antibiotic sensitivity test ampicillin and penicillin resistance and also similar to recorded by (Njagi et al., 2004)

At the last, we can conclude from the present work that *L.monocytogenes* are mainly food born pathogen that could contaminate poultry and milk products causing listeriosis. Sulphamethoxazole / trimethoprim is antibiotic of choice for listerial infection treatment followed by Gentamycin, Ceftazidime and Cephazoline *L.monocytogenes* express multidrug resistance to several antibiotics as Chloromphenicol , tetracycline ,Erythromycin, Ampicillin ,Penicillin and Clindamycin .All the isolated *L.monocytogenes* were CAMP Positive and produce β-zone of heamolysis .In PCR assured that the seven strains are *listeria monocytogenes* by detection of 16SrRNA gene and genotypic detection of resistance genes (Cat,MefA,Ampc,Aad6 and tet M).

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