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MOLECULAR CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* ISOLATED FROM SOME ABATTOIRS IN DAKAHLIA, EGYPT

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ABSTRACT

A total of 400 samples were collected from 100 slaughtered carcasses (cattle, buffalo, sheep and goat, 25 of each). Four samples from each carcass representing: muscle, liver, kidney and heart were collected for detection of *L. monocytogenes*. Also, 100 environmental samples representing water used in abattoir, knife swabs and swabs from abattoir walls before and after cleaning, (25 for each) and 100 fecal swabs from abattoirs' workers were randomly collected from different abattoirs located at Dakahlia governorate, Egypt. The Prevalence rate of *L. monocytogenes* in cattle carcasses was 3 (12%), 2 (8%) and 1(4%) in examined liver, kidneys and heart samples out of 25 examined samples for each organ, respectively and couldn't be detected in any of muscle samples. Meanwhile, in buffalo carcasses, the prevalence rate was 4 (16%), 2 (8%),1 (4%) and 1 (4%) in examined liver, kidneys, heart and muscle samples out of 25 examined samples for each organ, respectively. Meanwhile, in sheep carcasses, the prevalence rate of *L. monocytogenes* was 3 (12%), 2 (8%), 2 (8%) and 2 (8%) in examined liver, kidneys, heart and muscle samples out of 25 examined samples for each organ, respectively. Meanwhile, in goat carcasses, the prevalence rate was 4 (12%), 2 (8%), 2 (8%) and 1(4%) in examined liver, kidneys, heart and muscle samples out of 25 examined samples for each organ, respectively. In environmental samples, the prevalence rate was 28% from examined wall swabs before cleaning, 4% from examined wall swabs after cleaning, 4% from examined knife swabs and 4% from fecal swabs from abattoirs workers. Meanwhile, no *L. monocytogenes* was detected in water samples. Regarding pathogenicity test, death of all inoculated wining rabbits (23) as following 60% on 3rd day, 30% on 4th day and 10% on 5th day and *L. monocytogenes* was isolated from their organs. The isolates of *L. monocytogenes* were found to be virulent by using PCR assay incorporating *inl*A, *hly*A and *prf*A genes primers. The public health hazards as well as suggestive measures to reduce human listeriosis have been discussed.

Key words: *L. monocytogenes*, PCR, Virulence genes.

INTRODUCTION

Listeriosis in human poses a risk to pregnant women, newborn infants (called prenatal Listeriosis and represent one third of human listeriosis) and immunocompromised individuals (called adult Listeriosis and represent two third of human Listeriosis) and has a high mortality rate in these individuals of 20% to 30% (Gracieux *et al.*, 2003 and Rocourt *et al.*, 2000), healthy individuals also could develop milder form of gastrointestinal illness (Dalton *et al.*, 1997). In human, the illness may range from mild to severe sickness the sever form of human listeriosis are pressed as meningoencephalitis followed by septic infection and occasionally isolated organs involvement. Death is rare in healthy adults but can occur at a rate as high as 30% in persons at highest risk (Demetios *et al.*, 1996).

Occurrence of *L.monocytogenes* within slaughterhouses and meat processing facilities has been associated with environmental colonization, because of its ability to adapt and survive even on clean equipment and rooms (Lunde´n *et al.*, 2000). However, *L. monocytogenes* can enter through infected animals and raw meat or intermediate products processed by suppliers (Nesbakken *et al.*, 1996 and Sammarco *et al.*, 1997).

During slaughter, carcasses can become contaminated if they are exposed to small amount of intestinal content (Pal and Mahendra, 2015). In addition, animals are considered to be a part of food chain as important producers of meat and milk which provide high quality proteins and a key role in supplying calories (ESAP, 2001).

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Listeria monocytogenes was detected in the beef offal as slaughtered animals are recognized as reservoirs of foodborne pathogens (El-Gazzar and Sallam, 1997 and Mead, 2007).

There are several virulence genes so far identified in *L. monocytogenes*. These include the internalins (encoded by *inl* A, *inl* C, and *inl* J), listeriolysin O (LLO encoded by *hly* A), actin (*act* A), phosphatidylinositol-phospholipase C (PI-PLC encoded by *plc* A), *iap* (invasion associated protein encoded by *iap*), and virulence regulator (encoded by *prf*A). These virulence factors play significant role in the bacterial pathogenicity and infection outcome (Vázquez-Boland *et al.*, 2001 and Liu *et al.*, 2007).

Therefore, this study was designed to throw the light on the prevalence of *L. monocytogenes* in slaughtered cattle, buffaloes, sheep and goats in addition to some environmental samples representing water used in abattoir, wall swabs before and after cleaning beside fecal swabs from abattoir workers, as well as detection of virulent *L. monocytogenes* by amplification of different virulence associated genes of the isolated strains.

MATERIALS AND METHODS

Samples Collection:
A total of 400 examined samples were collected from 100 slaughtered carcasses (cattle, buffaloes, sheep and goats, 25 of each). Four samples from each carcasses representing: muscle, Liver, kidney and Heart were collected. Also, 100 environmental samples representing (25 samples from water used in abattoir and 50 wall swabs before and after cleaning and 25 knife swabs) and 100 fecal swabs from abattoirs' workers, were randomly collected from different abattoirs located at Dakahlia governorate, Egypt.

Samples preparations:
Samples were collected separately in sterile plastic bags, well identified and transported in an ice box (4 ^{o}c) to Animal health research institute, Mansoura Veterinary laboratory within 2 hrs. Collected Samples were cultured on the same day.

Part (I): Isolation and identification of *L. monocytogenes:*
The technique recommended by United Stated Department of Agriculture (USDA, 2002), Food Safety and Inspection Service (FSIS, 1989 and FAO, 1992) was adapted as following:

Enrichment procedure:
25 gm from each sample were aseptically weighted and added to 225 ml of Listeria enrichment Broth, University of Vermont Medium provided from Biolife (LEB UVM_I). The mixture was homogenized by using sterile mixture (New National) at high speed for 2 minutes.

The inoculated enrichment was incubated at 30°C for 24 hrs., then 0.1 of incubated (LEB UVM_1) were transferred to 10 ml (LEB UVM_{II}) and incubated at 30°C for 24 hours.

Isolation procedure:
A loopful from each of enrichment culture UVM_I and UVM_{II} broth were streaked onto palcam agar plates, then incubated at 30°C for 48 hours (Van Netten *et al.*, 1998 and Jemmi and Keusch, 1994).

Colonies showing morphological characters as dew drop-like, black colonies with brown hallow, or dark brown colonies 1-2 mm in diameter were streaked onto trypticase Soya agar supplemented with 0.6% yeast extract (TSA-YE) and incubated at 30°C for 24 hours till obtaining satisfactory pure separate colonies; which will be inoculated into semisolid agar and kept in refrigerator at 4^{o}C for further identification.

Identification of isolates:
Pure presumptive isolates were identified morphological and biochemically according to (FAO, 1992) using the classical tests.

Pathogenicity test for the isolated *L. monocytogenes* (Karin Hoelzer *et al.*, 2012):

(i) Experimental animals:
Wining rabbit weighting about 1kg (Number of inoculated rabbits were 23)

Bacterial suspension inoculum:
Each strain of well identified *L. monocytogenes* isolates from the examined samples were grown overnight in trypticase soya broth with 0.6 % yeast extract at 37°C, centrifuged and the sediment was resuspended in physiological saline (0.9%) and adjusted to the level used for inoculation 10^8 cells/ml. To know the concentration of the culture suspension, 1ml of the appropriate dilutions was spread onto the surface of prepared plate nutrient agar and incubated at 37 ^{o}C for 24 hours and then counted (Salwa Abd El-Ghafaar, 2007).

(ii) Animal inoculation:
Each rabbit was inoculated intraperitonealy with 0.1 ml of one of the bacterial suspensions, the inoculated rabbits were maintained under observation for evaluation of clinical signs and mortalities. Control rabbits were inoculated intraperitonealy with 0.1ml of physiological saline. Dead rabbits were sacrified and from each rabbit carcass, samples from liver, spleen and brain were collected and screened for presence of Listeria using Palcam medium.

Polymerase chain reaction (PCR) for *isolated L. monocytogenes* (Liu *et al.*, 2007):
Some pure isolates (10) of *L.monocytogenes* isolated from cattle, buffalo, sheep and goat carcasses and organs (liver, kidney and heart) were subjected to PCR assay at AHRI, Egypt.

DNA extraction (Liu *et al.*, 2007):
DNA extraction from some pure isolates (10) was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56ºC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer:
Primers used were supplied from Metabion (Germany) are listed in Table (1).

PCR amplification:
Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products:
The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. Gelpilot100 bpand 100 bp plus Ladders (Qiagen, Germany, GmbH) and generuler 100 bp ladder (Fermentas, Germany) were used to determine the fragment sizes.

The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
hlyA	**F:** GCA-TCT-GCA-TTC-AAT-AAA-GA	174	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Deneer and Boychuk, 1991**
	R: TGT-CAC-TGC-ATC-TCC-GTG-GT							
prfA	**F:** TCT-CCG-AGC-AAC-CTC-GGA-ACC	1052	94˚C 5 min.	94˚C 30 sec.	50˚C 50 sec.	72˚C 1 min.	72˚C 10 min.	**Dickinson *et al.*, 1995**
	R: TGG-ATT-GAC-AAA-ATG-GAA-CA							
inlA	**F:** ACG-AGT-AAC-GGG- ACA-AAT-GC	800	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	**Liu *et al.*, 2007**
	R: CCC-GAC-AGT-GGT- GCT-AGA-TT							

RESULTS

Results are illustrated in Tables (2-4) and Figures (1-3)

Table 2: Prevalence of *L. monocytogenes* in slaughtered carcasses and their offal:

Type of examined samples	Total no. of examined samples	Total positive samples		Muscle		Liver		Kidney		Heart	
		No.	%*	No.	%**	No.	%**	No.	%**	No.	%**
Cattle	100 (25 for each organ)	6	6%	-		3	12%	2	8%	1	4%
Buffalo	100 (25 for each organ)	8	8%	1	4 %	4	16%	2	8%	1	4%
Sheep	100 (25 for each organ)	9	9%	2	8%	3	12%	2	8%	2	8%
Goat	100 (25 for each organ)	9	9%	1	4%	4	16%	2	8%	2	8%

* % : Percentages calculated according to the number of samples examined / species (100).
**% : Percentages calculated according to the number of samples examined / organ (25).

Table 3: Prevalence of *L. monocytogenes* in the environmental samples and fecal swabs from abattoirs' workers:

Type of examined samples	No. of examined samples	Positive samples	
		No.	%*
Water	25	-	-
Wall swabs before cleaning	25	7	28
Wall swabs after cleaning	25	1	4
Knife swabs	25	1	4
Fecal swabs	100	4	4

* % : calculated according to the number of samples examined from each source (25 samples from each source and 100 fecal swabs)

Table 4: Pathogenicity test in wining rabbits:

No. of injected rabbit	Control +ve injected with normal saline	No. of rabbits injected with L.M*	No. of dead rabbits injected with L. M*			No. of dead rabbits injected with normal saline (+ve control)		
			3rd day	4th day	5th day	3rd day	4th day	5th day
23	**3**	**20**	**12**	**5**	**3**	**0**	**0**	**0**
			60%	**25%**	**15%**			

*****L.M: *Listeria monocytogenes*

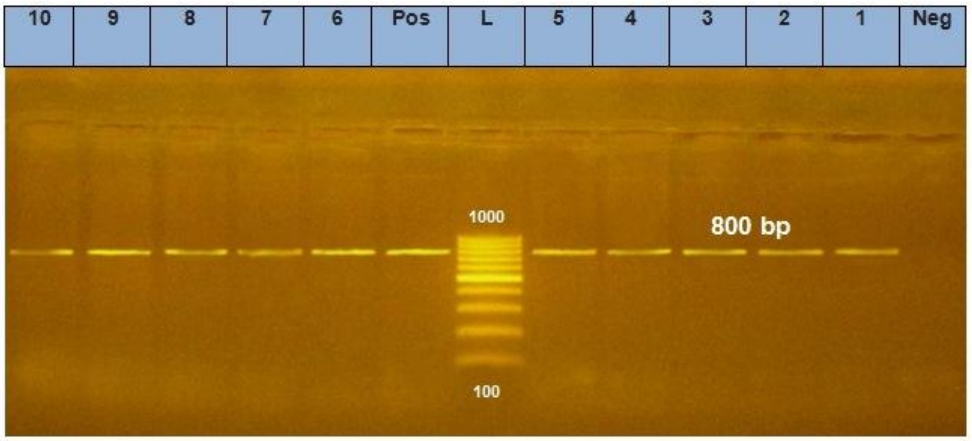

Figure (1): Agarose gel electrophoresis of *inl*A amplicons obtained from *L. monocytogenes* isolates.
L: 100 – 1000 bp ladder
1-10: Suspected *L. monocytogenes* DNA from the examined samples.

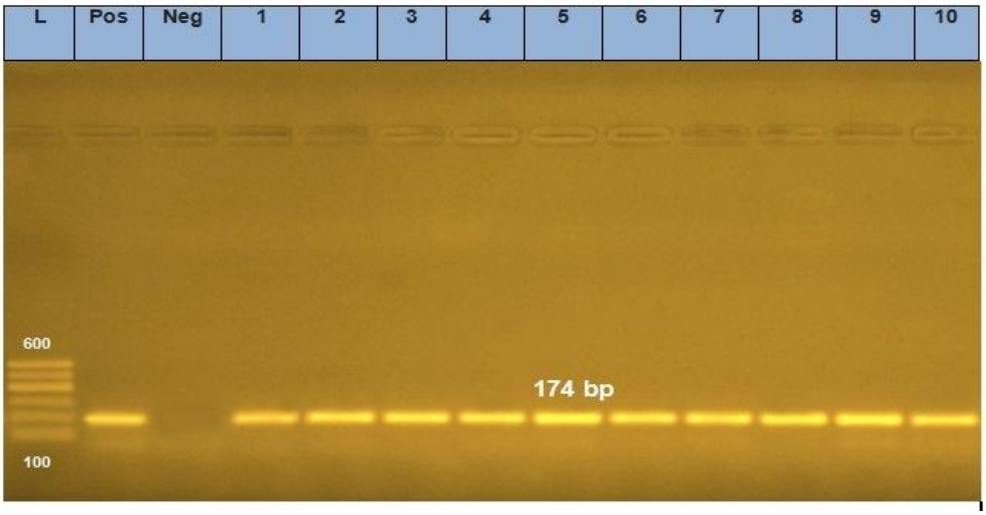

Figure (2): Agarose gel electrophoresis of *hly*A amplicons obtained from *L. monocytogenes* isolates.
L: 100 – 1000 bp ladder
1-10: Suspected *L. monocytogenes* DNA from the examined samples.

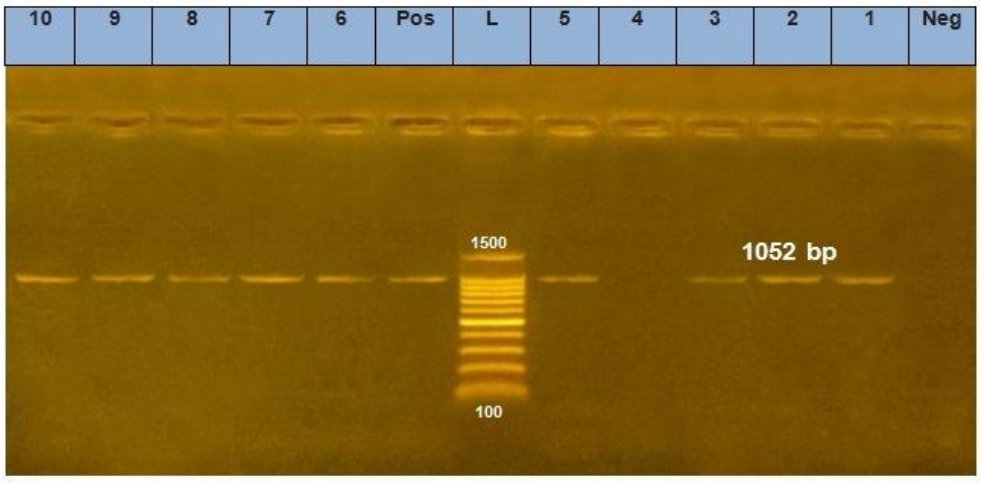

Figure (3): Agarose gel electrophoresis of *Prf*A amplicons obtained from *L. monocytogenes* isolates.
L: 100 – 1000 bp ladder
1-10: Suspected *L. monocytogenes* DNA from the examined samples.

DISCUSSION

It is evident from the results achieved in Table (2) that liver samples had the highest prevalence of *L. monocytogenes* 3 (12%), followed by kidney samples 2 (8%), and heart samples 1(4%) and no *L. monocytogenes* were detected in muscle samples. Such findings substantiate, what has been reported by El-Gazzar and Sallam (1997) and Buncic (1991) who reported that *L. monocytogenes* not demonstrated in the deeper parts of the muscles tissue from 12 beef carcasses all harboring Listeria in lymph nodes.

Regarding the examined samples of buffalo carcasses, the liver samples had the highest prevalence of *L. monocytogenes* 4 (16%), followed by kidney samples 2 (8%), then heart and muscle samples 1 (4%) for each. Such finding nearly similar to that reported by Chaudharia *et al.* (2004) and Jalali and Abedi (2008). The occurrence of *L. monocytogenes* in beef is described in several countries, emphasizing the contamination during the processing to the final and ready-to-eat products (Cordano and Rocourt 2001; Rorvik *et al.,* 2003; Mena *et al.,* 2004 and Uyttendade *et al.,* 1999).

Regarding the examined samples of sheep carcasses, the liver samples gave the highest prevalence of *L. monocytogenes* 3 (12%) followed by heart, kidneys and muscles samples 2 (8%) for each, out of 25 examined samples. Such findings agree with that reported by Vanderlinde *et al.* (1998) and Jalali and Abedi (2008).

Of the 25 examined samples of goat carcasses, the liver samples gave the highest prevalence of *L. monocytogenes* 4 (16%) followed by heart and kidney samples 2 (8%) for each then muscle samples1 (4%). Such finding were in agreement with that reported by Barbuddhe *et al.* (2000) and Dhary Alewy Al-mashhadany *et al.* (2016).

The difference in the isolation percentages may be due to difference in geographic distribution of Listeria, variations in animal husbandry and feeding practices or variation in methods of isolation (WHO, 1988).

The data represented in Table (3) showed that the Prevalence of *Listeria monocytogenes* in environmental samples was 28%, 4% and 4% from examined wall swabs before cleaning and after cleaning and knife swabs, respectively and was 4% in fecal swabs from abattoirs workers. Meanwhile, no *Listeria monocytogenes* was detected in water samples. Such findings agree with that reported by Miettinen *et al.* (2001), Norton *et al.* (2001) and Suihko *et al.* (2002).

The results of pathogenicity tests of isolated *L.monocytogenes* (Table 4) showed that rabbits inoculated by isolated *L. monocytogenes* were died within 2–5 days except control ones which remain alive without any clinical symptoms and in postmortem examination showed hemorrhagic foci in liver, spleen and brain of all dead rabbits. Re-isolation of *L. monocytogenes* from died rabbits in pure culture were carried out from brain and internal organs (liver and spleen). Stelma *et al.* (1987) and Tabouret *et al.* (1991) reported that pathogencity test must go parallel with the traditional method of culturing and identification of *Listeria monocytogenes*.

Many *L. monocytogenes* strains were naturally virulent, others were avirulent and unable to establish an infection within mammalian hosts (Jaradat *et al.,* 2002 and Liu *et al.,* 2003). So, it was of great concern to distinguish between virulent and avirulent strains for effective control and prevention measures of listeriosis.

PCR had proved to be an effective method for the detection of virulent *L. monocytogenes* by amplification of different virulence associated genes (Jaradat *et al.,* 2002).

In the present study, a PCR assay was carried out for detection of virulent *L. monocytogenes* by amplification of different virulence associated genes. All tested strains were positive for the *inl*A *and hly*A genes. While nine strains out of ten strains were positive for *prf*A as shown in Figures (1, 2 and 3, respectively). Similar results were documented by Vázquez-Boland *et al.* (2001), Liu *et al.* (2007), Mammina *et al.* (2009), Ahmed *et al.* (2014) and Henriques *et al.* (2017) who identified these virulence associated genes in the isolated *L. monocytogenes* from different sources.

CONCLUSION AND RECOMMENDATIONS

- *Listeria monocytogenes* is widely distributed in the environment, consequently slaughterhouses usually contaminated with this organism resulting in the contamination of meat and organs during evisceration.
- Liver is superior to other organs in harbouring *L. monocytogenes* in carcasses of cattle, buffalo, sheep and goat while meat of cattle was free from *Listeria monocytogenes*. Other organs (kidney and heart) harboured the organism in low extent.
- The virulence of *L. monocytogenes* is due to presence of virulence genes (*inlA, hlyA* and *prfA*) which could be detected by PCR as a powerful, rapid and accurate test.
- Strict hygienic measures should be applied within the slaughterhouses to prevent contamination of carcasses with *L. monocytogenes.*

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التوصيف الجزيئى لليستيريا مونوسيتوجينيس المعزولة من بعض مجازر محافظة الدقهلية بجمهورية مصر العربيه

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تم تجميع ٤٠٠ عينه من ١٠٠ ذبيحه من الأبقار والجاموس والأغنام والماعز (٢٥ عينه من كل فصيله)، ٤ عينات من كل ذبيحه تمثل العضلات والكبد والكلي والقلب تم تجميعها. وكذلك تم تجميع ١٠٠ عينه بيئيه تمثل المياه المستخدمه في المجازر ومسحات من السكاكين ومسحات من جدران المجازر قبل وبعد التنظيف (٢٥ عينه من كل نوع) و١٠٠ عينه تمثل مسحات شرجيه من بعض العاملين بهذه المجازر من اجل معرفة مدى تواجد ميكروب الليستيريا مونوسيتوجينيس. وهذه العينات تم تجميعها بشكل عشوائي من مجازر مختلفه بمحافظة الدقهليه بجمهورية مصر العربيه. هذا وقد اوضحت النتائج ان نسبة عزل ميكروب الليستيريا مونوسيتوجينيس من ذبائح الأبقار كانت ١٢ % و ٢% و ٤% في عينات الكبد والكلي والقلب علي التوالي في ٢٥ عينه من كل عضو تم فحصها ، ولم يتم عزل ميكروب الليستيريا مونوسيتوجينيس من أي من عينات العضلات .بينما في ذبائح الجاموس كانت نسبة عزل ميكروب الليستيريا مونوسيتوجينيس ١٦% و ٨% و٤% و٤% في عينات الكبد والكلي والقلب والعضلات علي التوالي في ٢٥ عينه من كل عضو تم فحصها. بينما في ذبائح الأغنام كانت نسبة عزل ميكروب الليستيريا مونوسيتوجينيس ١٢% و ٨% و٨% و ٨% في عينات الكبد والكلي والقلب والعضلات علي التوالي في ٢٥ عينه من كل عضو تم فحصها. بينما في ذبائح الماعز كانت نسبة عزل ميكروب الليستيريا مونوسيتوجينيس ١٢% و ٨% و٨% و ٤% في عينات الكبد والكلي والقلب والعضلات علي التوالي في ٢٥ عينه من كل عضو تم فحصها. كما تم عزل ميكروب الليستيريا مونوسيتوجينيس بنسبة ٢٨% و٤% و٤% من عينات المسحات المأخوذه من جدران المجازر قبل وبعد التنظيف ومن مسحات السكاكين علي التوالي في ٢٥ عينه من كل مصدرتم فحصها وبنسبة ٤% من عينات المسحات الشرجيه المأخوذه من بعض العاملين بهذه المجازر في ١٠٠ عينه تم فحصها. ولم يتم عزل هذا الميكروب من عينات المياه. وبدراسة ضراوة المعزولات علي صغار الأرانب أوضحت النتائج نفوق جميع الأرانب المحقونه وعددها ٢٣ أرنب كالتالي: ٦٠% في اليوم التالت و٣٠% في اليوم الرابع و١٠% في اليوم الخامس من الحقن. كما أكدت نتائج اختبار انزيم البلمره المتسلسل احتواء المعزولات علي بعض جينات الضراوه مثل *inlA*، *prfA* , *hyl*A.