Prevalence of Listeria Species in Fresh and Frozen Pigeon Carcasses with Studying Effect of Nigella Sativa Oil on Isolated *L. monocytogenes*

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Abstract: A total of 250 examined samples from 100 pigeon carcasses "50 fresh and 50 frozen" representing 150 fresh pigeon samples (50 skin, 50 muscles and 50 liver) and 100 frozen pigeon samples (50 skin and 50 muscles) were randomly purchased from poultry shops as well as supermarkets in Dakahlia Governorate, Egypt, and examined for the presence of Listeria species. The overall prevalence rate of *Listeria* species were 21.3% and 35% in fresh and frozen pigeon samples, meanwhile *L. monocytogenes* were 4% and 9% respectively. The other *Listeria* species (*L. innocea*, *L. grayi*, *L. murri* and *L. wellshomeri*) were isolated in an incidence of 8%, 4%, 3.3% and 2% respectively from fresh pigeon samples. From frozen pigeon samples, same *listeria* species were isolated in addition to *L. ivanovi* in an incidence of 14%, 6%, 3%, 2% and 1% respectively. The highest incidence of *Listeria* species was recorded in skin samples of both fresh and frozen pigeon samples as 30% and 44% respectively. The most predominant isolates was *L. innocea* 8.79% and 14% from fresh and frozen samples respectively. Fifty isolate of *L. monocytogones* were confirmed by PCR test, and pathogenisity to white mice of these isolate proved to be fetal for all inoculated experimental animals. Effect of *Nigella sativa* oil in a concentration of 10% after immersion for 2 minutes result in reduction CF 68% of inoculated *L. monocytogenes* in pigeon carcasses. The public health importance was discussed.

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1. Introduction:

Poultry meat are considered a good source of tasty easily digested and high biological value protein fat, minerals which are essential for growth and health of human being. Although most poultry meat come from chickens, significant amount of meat are produced from pigeon in Egypt and other countries in addition to the pigeons are acceptable and palatable to the most population. Listeriosis in human poses a risk to pregnant women, new born (called prenatal liseriosis) and immunocompromised individuals compresses two third of human listeriosis and has a high mortality rate in these individuals of 20% to 30% ***(Rocourt et al., 2000 and Gracieux et al., 2003)***. Healthy individuals also could develop milder from of gastrointestinal illness ***(Dalton et al., 1997)***.

Listeria species are gram-positive, short, non-sporing small rods, catalase positive and facultative anaerobes non capsulated, all closely related including *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, *L. murrayi* and *L. seeligeri* ***(ICMSF, 1996)***.

L. monocytogenes was the major pathogenic species in both man and animal ***(Mclauchlin and Jones, 1999)***. Although ***Cocolin et al. (2002)*** recorded that haemolytic species of listeria (*L. ivanovii and L. selligeri*) in addition to *L. welshimeri* were pathogenic to human. Recent outbreaks occur in Canada and Denmark due to ingestion of ready to eat meat and beef meat.

Pigeon Carcasses can be contaminated with some food borne bacteria during slaughtering defeathering, evisceration and other preparation process. One of the most important incriminated pathogens is *listeria* species.

Nigella sativa is a herbeceouse plant, whose seeds (black seed) have been used as spice and condiment in foods in the Middle East. *Nigella sativa* oil (black seed oil) could potentially be used to inhibit *L. monocytogenes* ***(Manojkumar, 2005)***.

The present study was done to determine prevalence of *listeria* species in pigeon meat (fresh and frozen) and effect of *Nigella sativa* oil on the isolated *L. monocytogenes* strains.

2. Material and Methods:

Collection and preparation of samples:

A total of 250 samples from 100 pigeon carcasses (50 fresh and 50 frozen) representing 150 fresh pigeon samples (skin, muscles and liver "50 for each") and 100 frozen pigeon samples (skin and muscles "50 for each") were randomly purchased from poultry shops and supermarkets in Dakahlia Governorate, Egypt.

Collected samples were well identified and separately packed in a sterile package and transported under refrigeration in ice box to the laboratory, without undue delay where they were screened for isolation and identification of exited listeria species. Skin samples including inner and outer surface of neck and coloca, meanwhile muscle samples were taken from breast and thigh muscle.

Isolation of listeria spp.:

The technique recommended by ***USDA, FSIS (1989)*** and ***FAO (1992)*** was adapted as following:

Enrichment procedures:

A portion of 25gm of each sample was homogenized with 255 ml of UVM, enrichment broth using mixer at high speed for 2 minutes and incubated for 24 hours, subsequently 0.1ml of incubated UVM I broth was transferred to 10ml UVM II broth then incubated at 30°C for 24 hours.

A loopful from each of enrichment cultured (UVM_1 and UVM_2) broth were streaked onto PALCAM agar plates then incubated at 30°C for 24 hrs. ***(Vannetten et al., 1998)***. Typical colonies were removed for further testing by inoculation on either trepticase soya agar or semisolid agar medium and kept in refrigerators at 4°C.

Identification of isolates:

Pure presumptive isolates were identified according to ***FAO (1992)*** by using Gram's stain, motility, catalase, oxidase, blood haemolysis sugar fermentation (Xylose, rhammna).

DNA extraction:

DNA extraction of the suspected colonies was performed according to the manufacturer guidelines using Bacterial DNA Extraction Kit (Spin-column) (**Bio Teke Corporation**). The suspected colonies obtained by cultural methods were re-suspended in nutrient broth and incubated at 37°C for 24 hours, according to Kit Manufacture's instruction.

Multiplex PCR for detection of virulence factors of *L. monocytogenes* genes:

Purified DNA of the suspected colonies was subjected to a multiplex PCR for the identification of *L. monocytogenes* and also for the detection of Internalin virulence factors according to ***Liu et al., (2007)***. Oligonucleotide primers synthesized by BioTeke Corporation (Canada) were used for the amplification of *L. monocytogenes* Internalin genes *in lA, in lC and in lJ. The in lA* primers were intended for species-specific recognition, and the *in lC* and *in lJ* primers were designed for virulence determination of *L. monocytogenes*. Table (1) shows the sequence of the primers and the expected product sizes. The reaction was performed in 25 µl reaction volume containing 12.5 µl of readymade 2 x PCR master mix (Bio Teke Corporation) and 40 pmol each *in lA*, 30 pmol each *in lC* and 20 pmol each *in lJ* primers and 2 µl of the purified DNA. A reaction mixture with no added DNA was run in the PCR as a negative control.

The reaction conditions consisted of one cycle of 95°C for 2 min followed by 30 cycles of 94°C for 20 sec, 55°C for 20 second 72°C for 50 sec and a final cycle of 72°C for 2 min. The reaction was carried out in Primus, MWG-Biotech Thermal Cycler. Amplification products were resolved in 1.2% (w/v) agarose gels along with 100 bp molecular weight ladder (BioTeke Corporation). The agarose gel was prepared in 1 x TBE (89 mM Tris- Borate, 2 mM EDTA, and pH 8.3) stained with 5 uM ethidium bromide. The gels were run in IxTBE, 5 µM ethidium bromide for at least 45 minutes at 100 volts and then visualized under Ultra Violet light of ultraviolet transilluminator (Spectroline).

Table (1): Primer sequences and expected product sizes of the multiplex PCR (Liu et al., 2007)

Gene	Primer sequence (5'→3')		Expected product size (bp)
inlA	*inlA-Forward*	ACGAGTAACGGGACAAATGC	800 bp
	inlA-Reverse	CCCGACAGTGGTGCTAGATT	
inlC	*inlC-Forward*	AATTCCCACAGGACACAACC	517bp
	inlC-Reverse	CGGGAATGCAATTTTTCACTA	
InlJ	*inlJ-Forward*	TGTAACCCCGCTTACACAGTT	238 bp
	inlJ-Reverse	AGCGGCTTGGCAGTCTAATA	

Pathogenicity test:

Female Swiss white mice weighting 18-20 gm were inoculated intraperitonealy with 0.1 ml of bacteria (suspensions)(10^8 m.o/ ml), control one were inoculated with 0.1ml phosphatase saline. Inoculated mice were maintained under observation for evaluation of clinical signs and mortalities. The dead mice were scarified and from each one, liver spleen and brain were collected and screened for listeria using PALCAM medium.

Effect of *Nigella sativa* oil 10% on isolated *L. monocytogenes* in experimentally contaminated pigeon carcasses:

The well morphological, microscopical, biochemical and tested by both PCR test and pathogenicity test strain of *L. monocytogenes* isolated from previous samples used for carrying out these experiment.

Experimental inoculation of *L. monocytogenes*:

Ten pigeon carcasses free from listeria were inoculated by dipping in bacterial suspension contain 10^7 cell/ml for 5 minutes then allow to dry and attachment with Bacterial cell in refrigerators for 10 minutes.

These inoculated samples were immersed in 10% *Nigella sativa* oil for 2 minutes (obtained from El-Gomheria Company for Chemical, Mansoura City). Control samples were immersed in distilled water for 2 minutes.

Listeria monocytogenes count was done immediately after treatment using plat count technique according to ***APHA, (1992)***.

3. Results:

Table (2): Prevalence of listeria species among the examined fresh pigeon carcasses

Type of samples	*No. of collected samples*	*No. of listeria isolates*		*Typing of isolated Listeria species*									
				Listeria monocytogenes		*Listeria innocaua*		*Listeria grayi*		*Listeria murri*		*Listeria welshemari*	
		No	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Skin	50	15	30	3	6	5	10	3	6	2	4	2	4
Muscle	50	8	16	2	4	3	6	1	2	1	2	1	2
Liver*	50	9	18	1	2	5	10	2	4	2	4	1	2
Total	150	32	21.3	6	4	13	8.1	6	4	5	3.3	4	2.6

*One of sample in liver contain mixed infection with *L. innocoua* + *L. welshemari*

Table (3): Prevalence of listeria species among the examined frozen pigeon carcasses

Type of samples	*No. of collected Sampl es*	*No. of listeria isolates*		*Typing of isolated Listeria species*											
				L. monocytogenes		*L. innocaua*		*L. grayi*		*L. murri*		*L. welshemari*		*L. ivanovii*	
		No	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
Skin	50	22	44	6	12	7	14	3	6	2	4	3	6	1	2
Muscle	50	13	26	3	6	4	8	3	6	1	2	2	4	-	-
Total	100	35	35	9	9	11	11	6	6	3	3	5	5	1	1

Table (4): Effect of 10% Nigella sativa on isolated L. monocytogenes

Before Treatment	*After treatment*				*Reduction*	
	Minimum	*Maximum*	*Mean*	*SE**	*Count*	*%*
10^7	3.5×10^6	5.3×10^6	3.2×10^6	4.1×10^6	6.8×10^6	68

***Standard error**

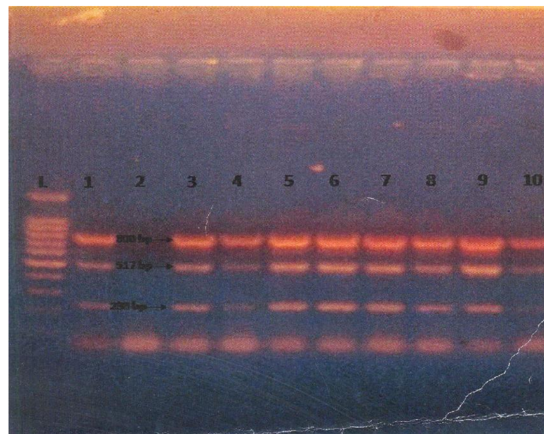

Figure (1): Agarose gel electrophoresis of inlA, inlC and inlJ amplicons obtained from *L. monocytogenes* suspected isolates (L: 100bp ladder, 1: positive control of *L. monocytogenes*, 2: negative control, 3 – 10: suspected *L. monocytogenes* DNA from the examined samples)

4. Discussion:

Listeria is a serious threat to food safety and ranks them among the food born microorganisms that most concern the food industry. Listeria is widely distributed in the environment and frequently contaminated the food. Among all species in the genous listeria, only *L.*

monocytogenes has been implicated in serious human illness ***(Blakova et al., 2004)***.

The results reported in table (2) revealed that Listeria species were isolated from 32 (21.3%) out of 150 examined samples of skin, muscle and liver of fresh pigeon carcasses. These results were higher than the reported by ***Rahimi et al.,(2012)*** and lower than that reported by ***Proietti et al., (2000)***. *L. monocytogenes* were identified from 6 (4%) of these samples. The most predominant isolated were *L. innocua* 13 (8.1%) followed by *L. grayi* 6 (4%), *L. murri* 5 (3.3%) and *L. wellshemeri* 4 (2.6%).

The highest prevalence rate of *Liseria* species and *L. monocytogenes* were isolated from skin samples 15 (30%) and 3 (6%) respectively. Furthermore, *L. innocoua*, *L. grayia*, *L. murri* and *L. wellshemery* were isolated from 5 (10%), 3 (6%), 2 (4%) and 2 (4%) respectively.

The obtained results declared that 8 (16%) and 9 (18%) of muscles and liver samples harbored *Listeria* species, while 2 (4%) and 1 (2%) were harbored *L. monocytogenes* respectively. Other *listeria* species could be isolated and identified as *L. innocua* (6%), *L. grayi* (2%) and *L. murri* (2%) in examined muscle samples; meanwhile in liver samples *L. innocua*, *L. grayi*, *L. murri* and *L. welshemari* were 10%, 4% and 2% respectively.

The occurrence of *lisetria* species in frozen pigeon samples are present in *table (3)* which declared that *Lisreria* species and *L. monocytogenes* more frequently isolated from skin (44%) and (12%) followed by liver samples (26%) and (6%), furthermore other *Listeria* species including *L. innocoua*, *L. grayi*, *L. murri*, *L. wellshemari* and *L. ivanovii* were recovered from 14%,6%,4%,6% and 2% from skin samples, meanwhile, in muscle samples they were 8%, 6%, 2%, 4% and 0% respectively. It is obevious from the aforementioned results of the examined frozen pigeon samples were more contaminated than that of fresh pigeon samples, these may be attributed to ability of *Listeria* species to grow and multiple in refrigeration temperature ***(Blakova et al., 2004)***.

The high prevalence rate of L. species from skin samples then both liver and muscle may be attributed that presence of wrinkles and irregulation of the skin provide physical protection to bacteria ***(Ellerbroke et al., 1996)****.while* ***Capita et al., (2002)*** reported that poultry can harbour *L. monocytogenes* in their intestinal tract and as such are potential source of contamination.

In the present study it is evident that all isolated and identified *L. monocytogenes* caused 100% mortality of inoculated white mice at 3rd and 4th day post inoculation. On postmortem examination hemorrhagic foci in liver, spleen and brain of all dead mice. Also the organism was reisolated from these organs. These findings were in harmony with the reported by ***Lammerding et al., (1992).***

The wide application of nucleic acid amplification techniques and the increasing industrial interest lowered rapid method has led to the development and application of PCR based methods for detection of microbial pathogens is food ***(Germini et al., 2009)***. Analyzing the PCR profiles 15 *Listeria monocytogones* isolates showed inlA, inlC and inlJ (100bp) which is specific for *L. monocytogenes*. Thus *L. monocytogenes* were confirmed in previously examined samples in *Figure (1).*

From the results presented in *table (4)* its evident that *L. monocytogenes* population were reduce from 10^7 cfu/cm^2 of the inoculated chicken skin samples by *Nigella sativa* oil 10% for 2 minutes to 3.2×10^6 with a percentage of 68%. It is clear that 10% Nigella sativa oil had a significant influence on isolated *L. monocytogenes*, such results adequately consistent with ***Manojkumar, (2005)***.

Listeria in human and animal usually associated with *Listeria monocytogenes* and *Listeria seeligeri* ***(Mclauchlin et al., 2004)***. However there is some evidence for very rare infections caused *Listeria innocuain* in domestic animals ***(Walker et al., 1994)***. The other species of *Listeria* have not been reported to cause disease. The widely distribution of Listeria species in nature due to its ability to tolerate high concentration of salts and relatively low pH and multiply at refrigeration temperature are considered a serious thereat to food safety and rank them among the microorganisms that most concern the food industry. *Listeria monocytogenes* is the only species of the genus *Listeria* that involved in known food born out break of serious diseases.

The public health importance of *Listeria monocytogenes* has been recorded by many authors ***(Hof, 2001 and Longhi et al., 2003)***. In order to minimize human Listeriosis, foods should be cooked to an internal Temperature of 70°C for more then 20 minutes to ensure distraction of *L. monocyt*ogenes. Reheat cooked food thoroughly (70°C) immediate aseptic packaging of the finished product to avoid post processing environmental contamination. Proper cold storage of meat and meat products (freezing -18°C) and proper personal hygiene of food handlers is advisable ***(Mahmoud et al., 2003)***.In concentration 10% and immersion for 2 minutes results in reduction CP 68 % of inoculated *L. monocytogenes* in pigeon carcasses.

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