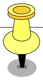

Effect of Listeria monocytogenes on Food and Water Sources in Buffaloes' Farms

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Effect of Listeria monocytogenes on Food and Water Sources in Buffaloes' Farms

Abstract:

Listeria monocytogenes from Listeria genus, which as saprophytes, but causes systemic infections as pathogenic bacteria. Listeria monocytogenes is widely spread in environment, caused infection of farm animals during fields grazing leading to contamination by wildlife, or manure. There was a close relation between improperly farm environment originated from contaminated crops and listeriosis. It causes animals infection with is not associated with clinical symptoms then the animal is flaking Listeria monocytogenes in feces. Listeria species' identification is a serious to gain the ecological Listeria virulence influence. The samples understudy consisted of animal stool, mattress, ration and water (n = 30) were for each. All samples were exposed to bacterial check. The results obtained that *Listeria* species was isolated with a general occurrence of 33.3%. The stools had 17.5%, mattress 7.5%, ration 5.0%, and water 3.3%. Meanwhile the occurrence of *Listeria monocytogenes* was overall 20.0% included (10.0%, 5.0%, 2.5%, and 2.5 %) as the same aforementioned samples. The PCR-technique was applied for detection of *Listeria monocytogenes* virulence genes using primers groups. Those genes were *hlyA* (hemolysin A gene), *inlA* (internalin A), *prfA*. The results obtained the present of all virulence genes. *Listeria monocytogenes* susceptibility to antimicrobial agents usually used for farms treatment was determined for animal stools *Listeria monocytogenes* isolates. The results showed that the generally sensitive rate was 70.0%. The100.0% was sensitive to Ampicillin, Amoxicillin, Erythromycin, and Tetracycline followed by Ofloxacin 75.0%. But the 50.0% was sensitive to Penicillin G, Cephalexin, and Gentamycin, finally Lincomycin

was resistant. It was concluded that the *Listeria monocytogenes* were isolated from understudy samples as noticeable degrees. Also, virulence genes were presented in the isolated *Listeria monocytogenes*. That powerful proved the attendance of *Listeria monocytogenes* trendy a hidden contamination in animal farm. So, that could affect the animal health and animal productions. It was recommended that the undertake to follow the "Farm Health Conditions" and follow-up to decrease the microbial contamination. As well fix the animal vaccinations to maintain the animal health and livestock.

Key words: Listeria monocytogenes, Virulence gene, PCR-technique, inlA, inlB, hlyA.

Introduction:

Listeria bacteria are small Gram-positive rods, not forming spores or capsule **(Rodríguez-Melcón e*t al.,* 2022).** *Listeria monocytogenes* considered as pathogenic bacteria **(Humski *et al.,* 2022).** It was widely spread in environment, caused infection of farm animals during grazing. There was a close relation between improperly farm environment originated from contaminated crops and listeriosis in ruminants. Animal infection with *Listeria* either not related with clinical signs but the animal was able to feces bacterial shed **(Farag *et al.,* 2021).**
Founded by phenotypic and genotypic typical resemblances and changes, *Listeria* species have "*Listeria sensu lato*" and "*Listeria sensu strictu*" **(Chiara *et al.,* 2015 and Cao *et al.,* 2018).** *Listeria* sensu strictu contain *Listeria monocytogenes, Listeria* i*nnocua, Listeria seelgerii, Listeria welshimeri*, and *Listeria marthii*. All *Listeria* sensu strictu are grow below or at 4°C, give positive catalase, and motile at 30°C. *Listeria* sensu lato contain 11 species (**Weller *et al.,* 2015).** *Listeria* species are nature profuse and can live in all environmental stresses types. *Listeria monocytogenes* caused human listeriosis, lead to serious health

problematic, **(Fallah *et al.,* 2012).** They caused severe symptoms and high mortality 20%. *They* recognized to produce humans and animals disease (**Mazza *et al.,* 2015).**

The antimicrobial agents increased use in animal treatments and in food sorted was a significant factor in the appearance of antimicrobial-resistant bacteria. The antimicrobials dull use in animal food production for treatment and growth raise was a significant factor for the appearance of multidrug resistant microbial strains **(Odu and Okonko, 2017).**

Polymerase Chain Reaction-technique (PCR-technique) had a great potential to speed-up the detection of *Listeria monocytogenes* in food **(Trinh and Lee, 2018).** PCR-technique provided a powerful designing nucleic acid-based assays that were highly specific, sensitive, and quantitative **(Maffert *et al.,* 2017).** Initially, detection PCR-technique products were talented via gel electrophoresis **(Ghetas *et al.,* 2022).**

The current paper aim was assumed with the major target of isolating and identifying of *Listeria* species from various samples of animal source. Determining the *Listeria monocytogenes* virulence factors gene. Which was known as the hidden contamination that may lead to animal disease and could affect the animal and animal products as well determine the antimicrobial susceptibility drug.

Materials and Methods:

- **Samples Collection:** The current study was carried during (January – February) / 2022, that was on buffaloes' farms at Giza, Egypt. A total of 120 samples, including of animal stool, mattress, ration, and water (n = 30) were for each, that were used sterile swab. Then each swab was inoculated into "Trypticase Soya Broth" added by 0.6% "Yeast Extract" (TSB-YE) **(Farag *et al.,* 2021).**
- **Bacterial Isolation and Identification:** The swab was inoculated on "Palcam Agar Plate"; (Oxoid; CM0877B and

SR0150E), then was incubated at 35°C for 24-48 hours. The ISO 112090-1 food sampling protocol was used to detect *Listeria* cells. The same swab was placed in 20 ml of "Fraser Broth"; (Oxoid; CM0895B and SR0156E) and was vortex 30 second. Loopful from each broth inoculate on "Palcam Agar Plate" and as before. Th plates were examined for colonies morphology of *Listeria*. Its green color with black sunken center and black halo. The Gram Staining-technique, Motility test, biochemical and serological tests were performed **(Farag *et al.,* 2021).**

- **PCR-technique Application:** This was applied for *Listeria monocytogenes* virulence genes detection using Primer sequences sets. These genes were *hlyA* (hemolysin A gene), *inlA* (internalin A), and *prfA* (positive regulatory factor A), were used Primer sequences, amplicon size and PCR-technique program (Table 1). PCR-technique was applied following QIA amp DNA mini-kit orders (Catalogue no.51304), Dream Taq Green PCR-technique Master-Mix (2X) (Thermo Scientific) Cat No. K1081 and agarose gel electrophoreses **(Law *et al.,* 2015).**

  -DNA Extraction: QIAamp DNA Mini-kit (Qiagen, Germany, GmbH) with modifications manufacturer's recommendations. Sample suspension 200 µl was incubated with 10 µl proteinase K also 200 µl lysis buffer at 56°C to 10 min. Then 200 µl ethanol 100% was added to the lysate. The sample was washed and centrifuged following the kit. Nucleic acid was eluted with 100 µl elution kit buffer **(de Faria *et al.,* 2022).**

  -Oligonucleotide Primer: Primers used were full from Metabion (Germany); (Table 1), **(Govindkumar *et al.,* 2022).**

Table 1: Primer sequences of Listeria monocytogenes virulence genes detection

Target bacteria	Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
Listeria monocytogenes	inlA	ACG AGT AAC GGG ACA AAT GC CCC GAC AGT GGT GCT AGA TT	800	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	72°C 10 min.	(Liu et al., 2007)
	inlB	Ctggaaagtttgtatttgggaaa tttcataatcgccatcatcact	343	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 40 sec.	72°C 10 min.	
	hlyA	GCA-TCT-GCA-TTC-AAT-AAA-GA TGT-CAC-TGC-ATC-TCC-GTG-GT	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)

-Uniplex PCR-technique: The primers were used in 25 µl reply covering 12.5 µl Emerald Amp-Max PCR-technique Master-Mix (Takara, Japan), 1 µl apiece primer 20 pmol concentration, 4.5 µl water, and 6 µl DNA template. The response was doing in an Applied-biosystem 2720 thermal-cycler **(Aziz and Lafta, 2022).**

-Stx1, stx2 duplex PCR-technique: The primers were used in 50 µl response covering 25 µl Emerald Amp-Max PCR-technique Master-Mix (Takara, Japan), 1 µl apiece primer 20 pmol concentration, 13 µl water, and 8 µl DNA template. The reaction was doing in an Applied-biosystem 2720 thermal-cycler **(Bording-Jorgensen *et al.*, 2022).**

-Products of PCR-technique: That were detached 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 uniplex PCR-technique products and 40 µl duplex PCR-technique products were loaded in each gel slot. Gene ruler 100 bp ladder (Ferments, Thermo) and gel pilot 100 bp plus ladder (Qiagen, gmbh, Germany) were used to fragment sizes control. The gel was photographed by a gel documentation system (Alpha Inno-tech, Bio-metra) and data was analyzed via computer software **(Ghetas *et al.*, 2022).**

- **Antibiotics Sensitivity Test:** *Listeria monocytogenes* animal stool isolates were tested by the single diffusion method. The

isolate was spread on "Mueller-Hinton Agar"; (Oxoid Limited, UK). The ”Antibiotic Discs"; (Oxoid, Basing-stoke, Hamp-shire, UK), were distributed on the plate surface. The plates were reared at 25°C for 2-7 days and were measured halo zone around the discs and interpreted by “Clinical and Laboratory Standards Institute”; (CLSI); **(Nasrin *et al.,* 2022).**

- **Following the Bio-safety Procedures:** These works were doing in the specialized laboratory while conducting the experiments **(Al-Shabi and Abuhamdah, 2022).**
- **Data Analysis:** The results were arranged by “Simple Excel Program” **(Zaki *et al.,* 2021).**

Results and Discussion:

- **Incidence of *Listeria* species were isolated from understudy samples:** *Listeria monocytogenes* considered as one of the most insidious food-borne pathogens that primarily affects individuals at high risk resulting in a mortality rate 15-20%. *Listeria monocytogenes* can cross the placental fence and did foetus infection, causing unwary abortion, miscarriage, and even pre-term and neonatal infections in animals and humans **(Esposito *et al.,* 2021).** Human infection happens result of the ingesting of contaminated milk, dairy crops, and animal source food **(Garvey, 2019).**

  The results exposed that about one third about 33.3% of the understudy samples were found positive for *Listeria* species. This indicated *Listeria* species presence in unclear and unexpressive animal environment places as a contaminant. It was higher isolates from animal stool samples was 17.5%. Then followed by animal mattress was 7.5%, then animal ration was 5.0%, and final animal water was 3.3% (Table 2). This was the biggest evidence of the presence of *Listeria* species bacteria in the animal’s surrounding environment and

could easily transmitted to the animal itself. An infection may occur by these bacteria *Listeria* species. In Egypt as at 2018 from Dakahlia province, Egypt, the samples had *Listeria* species 17.1% in animal faeces, *Listeria monocytogenes* was 44.4% **(El-Gohary *et al.,* 2018).** At 2019 from Sharkia and Dakahlia Governorates, Egypt, the cows faeces samples had *Listeria* species about 8% **(EL-Naenaeey *et al.,* 2019).** In 2021 from Damanhour city, El-Behira Governorate, Egypt, the samples had 12.88% Listeria species **(Saleh *et al.,* 2021).** From North Coast and Desert Road, Egypt, at 2021, private buffaloes' farms revealed 16.7% *Listeria monocytogenes*, and 4% was from farm animals faeces, that indicated contamination **(Farag *et al.,* 2021).** This expresses the attendance of *Listeria* species bacteria in the animal's surrounding environment and is transmitted to it very easily to cause disease. Therefore, the health conditions of farms animal must be followed to reduce microbial contamination as *Listeria* species that may lead to disease in animals. That will affect an animal health, as the impact on animal production that will affect money income from animal farm **(Manyi-Loh *et al.,* 2018).**

Table 2: Incidence of Listeria species were isolated from understudy samples

Understudy samples 120	Positive samples	Percent
Animal stool (30)	21/120	17.5%
Animal mattress (30)	9/120	7.5%
Animal ration (30)	6/120	5.0%
Animal water (30)	4/120	3.3%
Total	**40/120**	**33.3%**

- **Incidence of *Listeria monocytogenes* confirmed biochemical and serology:** It was found that eight *Listeria monocytogenes* of the forty *Listeria* species isolates. However, half of *Listeria monocytogenes* were found in

animal stool as 10.0%. The other quarter was in animal mattress as 5.0%. The other quarter was divided between animal ration and animal water as (2.5% and 2.5%); (Table 3). Therefore, it was proved that *Listeria monocytogenes* were clearly present in the farm. This affected the animal health and causes bacterial contamination of the environment surrounding the animal. So, it can move again to food, water and the residence place of the animal, which could increase its numbers as contaminant **(El-Gohary *et al.,* 2018; EL-Naenaeey *et al.,* 2019; Saleh *et al.,* 2021; and Farag *et al.,* 2021).**

Therefore, it can pass to animal products and pass to other animals as infected agent, which given low quality products. This affects animal farm health. Therefore, important steps must be taken to preserve animal health through the health conditions of farms to preserve farm animals. That to reduce infection, raise the quality of animal products and increase financial income from the animal farm **(Ha, 2021).**

Table 3: Incidence of Listeria monocytogenes confirmed biochemical and serology

Positive samples for Listeria species 40	Positive samples	Percent
Animal stool	4/40	10.0%
Animal mattress	2/40	5.0%
Animal ration	1/40	2.5%
Animal water	1/40	2.5%
Total	**8/40**	**20.0%**

- **PCR-technique for *Listeria monocytogenes* virulence genes:** PCR-technique offers were a very powerful tool to elaborate specific, sensitive, and rapid detection methods for bacterial pathogens in food products, both clinical and environmental samples **(Hoorfar, 2011).** PCR-technique used as diagnostic exploring apparatuses for the presence of

virulence factor in three representative samples included *inlA*, *inlB* and *hlyA*. The results obtained the presence of the three virulence genes in two samples and one virulence gene only on one sample. It was worth to mention that *Listeria* species that was detected by Multiplex (m. PCR-technique); (Table 4, Photo 1 & 2). That was successfully amplified virulence factors *hylA* gene at specific base pair 456 **(El-Gohary *et al.*, 2018).** At 2019 the multiplex PCR-technique had been standardized by using primers to amplify *hlyA* gene and expected band at 370 bp were obtained **(El-Sayed *et al.*, 2019).**

PCR-technique was encoded by pathogenicity island (LIPI-1) **(Osman *et al.*, 2020).** In Sharkia and Dakahlia Governorates, Egypt at 2019, *Listeria monocytogenes* were established of *inlA* and *inlB* genes had 80% and 40%. Internalin (*A & B*) genes careful the virulence determination best stick **(EL-Naenaeey *et al.*, 2019).** During 2020 isolation of *Listeria* was done according to ISO 11290. Conventional PCR-technique was *hlyA* positive. Ethidium bromide stained agarose gel electrophoresis containing the PCR-technique products along 100bp DNA ladder (lane L). Gel Pilot 100 bp ladder (cat. no. 239035) provided by QIAGEN (USA). Number of bands: 6 and size range: 100-600 bp **(Ahmed *et al.*, 2020).** During 2021 Mansoura city, Dakahlia Governorate, Egypt, *Listeria monocytogenes* were 7% in samples, *hly* gene presented in 6 isolates **(El-Baz *et al.*, 2021).** In 2021 from Damanhour city, El-Behira Governorate, Egypt, *Listeria* species was 12.88%, *hylA* gene was 83.3% **(Saleh *et al.*, 2021).**

Table 4: PCR-technique for Listeria monocytogenes virulence genes

Listeria monocytogenes sample	inlA	inlB	hlyA
1	-	+	+
2	+	+	+
3	-	+	+

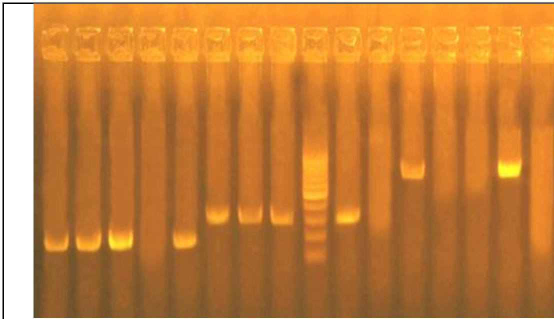

Photo 1: PCR-technique for Listeria monocytogenes virulence genes

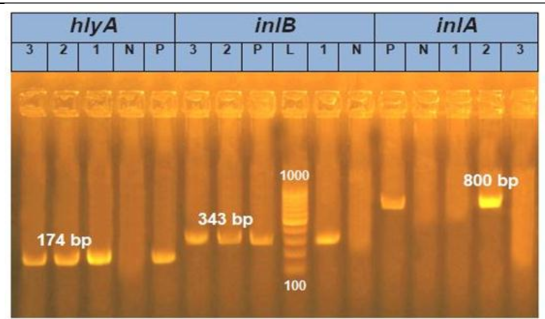

Photo 2: Explain PCR-technique for Listeria monocytogenes virulence genes

- **Antimicrobial susceptibility for *Listeria monocytogenes* were isolated from animal stool samples:** The experiment was done on *Listeria monocytogenes* the most common in the animal stool (Table 5). The whole sensitive rate was seventy percent 70.0%, and the non-sensitive rate was thirty percent 30.0%. The differential sensitivity was distribution of the antibiotics used. It was found that 100.0% was extra sensitive to Ampicillin (25µg), Amoxicillin (25µg), Erythromycin (15µg), and Tetracycline (30µg). Three quarters 75.0% was sensitive to Ofloxacin (10µg) only. Half 50.0% was sensitive to Penicillin G (10IU), Cephalexin (30µg), and Gentamycin (10µg). Finally, the non-sensitive 00.0% was to Lincomycin

(2µg) (**Ahmed *et al.*, 2020; El-Baz *et al.*, 2021; and Saleh *et al.*, 2021).**

Table 5: Antimicrobial susceptibility for Listeria monocytogenes were isolated from animal stool samples

Listeria monocytogenes isolates 4 Antibiotics (Concentration)	Sensitive isolates		Resistant isolates	
	Number	Percent	Number	Percent
Ampicillin AMP (25µg)	4/4	100.0%	0/4	0.0%
Amoxicillin AX (25µg)	4/4	100.0%	0/4	0.0%
Erythromycin E (15µg)	4/4	100.0%	0/4	0.0%
Tetracycline TE (30µg)	4/4	100.0%	0/4	0.0%
Ofloxacin OFX (10µg)	3/4	75.0%	1/4	25.0%
Penicillin G P (10IU)	2/4	50.0%	2/4	50.0%
Cephalexin CL (30µg)	2/4	50.0%	2/4	50.0%
Gentamycin CN (10µg)	2/4	50.0%	2/4	50.0%
Lincomycin L (2µg)	0/4	00.0%	4/4	100.0%
Mean	**2.8/4**	**70.0%**	**1.2/4**	**30.0%**

Conclusion:

It was concluded that the *Listeria monocytogenes* were isolated from understudy samples as noticeable degrees. Also, the virulence genes were presented in the isolated *Listeria monocytogenes*. That powerful proved the attendance of *Listeria monocytogenes* in a hidden contamination in animal farm. So, that could affect the animal health and animal productions.

Recommendation:

It was recommended that the undertake to follow the "Farm Health Conditions" and follow-up to reduce the microbial contamination. As well fix the animal vaccinations to maintain the animal health and livestock.

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Thanks, were sent to farm owners, Lab. technician, PCR-technique Dept.

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