Effect of Chemical and Environmental stress on Listeriolysin O production in *Listeria monocytogenes*

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Abstract:

Listeriolysin O (LLO) is a major virulence factor in *L. monocytogenes* that required for intracellular survival and establishment of infection in host cells. the current study, some chemical and environmental stress, including salt concentration and pH, effect on the production of listeriolysin O by pathogenic *L. monocytogenes* isolates was evaluated using quantitative microtiter plate hemolysis assay in trypticase soya broth medium (TSB). The activity of listeriolysin O was optimal at 0.5% NaCl, pH 7.0 to 7.5, and 37ºC, and in presence of oxygen. The hemolysis assay results showed that hemolysin activity reduces in acidic pH, where (pH 5.5: 24.5%), (pH 6.0: 33%), and (pH 6.5: 77.5%) hemolysis. In contrast, hemolysis assay results were showed enhances of hemolysin activity for *L. monocytogenes* isolates in alkaline pH, where, (pH 8.0: 122.5%), (pH 8.5: 150.5%), and (pH 9.0: 130%) hemolysis. These results appeared significant differences ($P \leq 0.05$) compared to optimal pH (7.0). In the other hand, hemolysis assay results with different salt concentration (NaCl) were showed increases in hemolysin activity in low salt concentration, where, (1%NaCl: 152%) and (2%NaCl: 143%). Whereas high level of salt concentration were showed reduce in hemolysin activity in sheep red blood suspension, where (3%NaCl: 70.3%) and (4%NaCl: 66%), (5%NaCl: 50%) and (6%NaCl: 42%), (7%NaCl: 33%) and

(8%NaCl: 23%), and (9%NaCl: 17%), with significant differences ($P \leq 0.05$) compared to optimal salt concentration (0.5). From these results, we concluded that acidic pH and alkaline pH, as well as different level salt concentrations, may be the effect on the activity of listeriolysin O, which responsible for the development of listeriosis in human.

Keyword: stress, Listeriolysin O, Listeria monocytogenes.

Introduction:

(+) gram pathogenic *L. monocytogenes* is a etiological agent of listeriosis disease , the disease transmitted by contaminated food associated with severe clinical signs such as meningoencephalitis ,abortion and meningitis in human and animals (Vazquez-Boland *et al*., 2001). *L. monocytogenes* produces many of virulence factors like (LLO) is considered a extracellular virulence factor produced by all pathogenic isolates of *L. monocytogenes* (Leimeister-Wachter *et al*., 1990). Infection of *L. monocytogenes* has four stages: intery, escape from a vacuole, nucleation of actin filaments, and transmitted to another cell (Gedde *et al*., 2000). Listeriolysin O as pore forming a protein which lysis of vacuole membrane play an important role in intracellular survival and proliferation of bacterial cells (Goebel and Kuhn, 2000). *L. monocytogenes* is grown influence its virulence and its virulence factor activity under environmental conditions under (Dallmier *et al*., 1990; Myers *et al*., 1993). *L. monocytogenes* has a salt-tolerant and has capable of growing at temperatures ranging from (1- 44°C) (18). Its grow at refrigerator temperatures, and at pH values from (5-9), and at salt concentrations up to 10% renders it a considerable threat to public health, as consumption of contaminated foods can result in serious disease

(listeriosis) (Farber and Peterkin, 1991).The present study was designed to determine whether different levels of sodium chloride (NaCl) and hydrogen ion concentrations (pH) had any effect on Listeriolysin O virulence factor activity of *L. monocytogenes.*

Materials and Methods:

Listeria monocytogenes isolates:

Listeria monocytogenes isolates were obtained from the laboratory of Microbiology, Faculty of Science, Al-Muthana University. *Listeria monocytogenes* isolates were tested demonstrate the β-hemolytic characteristics of *L. monocytogenes* has grown on blood agar and biochemical properties by using (API Listeria Kit. bioMérieux). As well motility test that characteristics of *L. monocytogenes* which grown on semisolid medium.

A quantitative microtiter plate hemolysis technique:

A triplicate of hemolysis technique was carried out from different colonies according to the method by (Sampathkumar *et al*., 2002). *L. monocytogenes* isolates were streaked on brain heart media then incubated at (37°C) for 24 hours, then a single colony inoculated into 5 ml of trypticase Soya broth (Difco) tubes that prepared in different levels of hydrogen ion concentrations (pH) ranged from (5.5 - 9.0). In another side, a single colony inoculated into 5 ml of trypticase Soya broth tubes were prepared with different sodium chloride concentrations (NaCl) ranged from (1 – 9%). Then all samples incubated at 37°C with shaking at (200 rpm) for 18 hours. Sheep red blood cells (SRBCs) suspension were prepared by using phosphate buffer saline at (1500 rpm) in the

tabletop cold centrifuge for 15 minutes, then diluted to obtain a final concentration of (3%) SRBCs suspension.

To measure the hemolytic activity to each *L.monocytogenes* isolates, 200µL bacterial cells growth were centrifuged at (11500 rpm) for 5 minutes in high-speed centrifuge to obtain a hemolysin-containing supernatant. A 200 µL of (PBS with 1 mM DTT pH 5.8) was added to the supernatant to make seven serial dilutions (1:2) in 1.5ml Eppendorf tubes, then, all tubes were incubated at 37C° for 30 minutes. After that, 200 µL of diluted SRBCs were added into each supernatant tube and then mixed gently by swirling the tubes. For positive control, 200 µL of 1% triton X was prepared and added to 200 µL of SRBCs to complete lyses of the SRBC. And for negative control, 200 µL of SRBCs were added into in 200 µL (PBS/1Mm DTT) without diluted supernatant, and then all tubes were incubated at 37C° for 60 minutes. All tubes centrifuged at (1000 rpm) for 5 minutes, then 200µl of the assay supernatant was taken and loaded into (Microtiter plate 96-well, U-bottom), and the hemolysis (haemoglobin release) is read at (420) nm absorbance by microtiter plate reader.

The following equation was used for calculation of hemolytic unit:

Hemolytic Unit = [(positive control O.D)-(negative control O.D) / 2]

Hemolysis percent = (ODs -OD-ve) / O.D+ve × 100%

Where, O.Ds = Optical Density.

Results:

Characteristics of *L.monocytogenes* isolates:

Listeria monocytogenes isolates were showed β-hemolytic colony on blood agar, and given umbrella like shape on a semisolid medium that demonstrates bacterial motility, figure (1). API- Listeria strip results were

read after incubated for 18 hours and results determine according to kit instruction, where, the isolates showed able to fermentation three sugar only (D-ArabitoL, L-Rhamnose, and MDG; Methyl-αD-glucopyranoside) whereas appeared unable to fermentation of other sugar tests such as (DIM; Enzymatic substrate, XYL; D-Xylose, RIB; D-Ribose, G1P; Glucose-1-Phosphate, and TAG; D-Tagatose) as well as showed able to hydrolysis of Esculin ferric citrate (ESC) , negative for (Differentiation *L. innocua* from *L. monocytogenes* (DIM)), and 4-nitrophenyl-αDmannopyranoside (αMAN). Figure (2) illustrates API- Listeria strip reaction before and after incubation.

Api- Listeria test before incubation

Api- Listeria test after incubation

Figure (1) Motility test showed umbrella like shape in semisolid medium

Figure (2) Api- Listeria strip biochemical tests before and after incubation.

Hemolysis Assay Results:

The hemolysis assay results showed that hemolysin activity is not similar at different pH levels, where, the acidic pH was showed reduce in hemolysin activity as (pH 5.5: 24.5%), (pH 6.0: 33%), and (pH 6.5: 77.5%) hemolysis compared to optimal pH (7.0-7.5). which given (pH 7.0: 97%) and (pH 7.5: 100%) hemolysis. In contrast to alkaline pH appeared enhances to hemolysis activity, where, (pH 8.0: 122.5%), (pH 8.5: 150.5%), and (pH 9.0: 130%) hemolysis (Table 1). In the other hand, hemolysis assay results in trypticase Soya broth with different sodium chloride concentrations (NaCl) were showed increases in hemolysin activity in low sodium chloride concentrations, where, (1%NaCl: 152%) and (2%NaCl: 143%). Whereas high level of sodium chloride concentrations were showed reduce in hemolysin activity in sheep red blood suspension, where (3%NaCl: 70.3%) and (4%NaCl: 66%), (5%NaCl: 50%) and (6%NaCl: 42%), (7%NaCl: 33%) and (8%NaCl: 23%), and (9%NaCl: 17%) (Table 2).

Table (1): The hemolysis assay results with different hydrogen ion concentrations

pH	Hemolytic Unit	Mean of O.D value	Hemolysis percent (100%)
5.5	1/ 8	0.76	24.5*
6.0	1/ 16	0.978	33*
6.5	1/ 32	1.909	70.3*
7.0	1/ 32	2.581	97
7.5	1/ 32	2.653	100
8.0	1/ 64	3.215	122*
8.5	1/ 64	3.921	150.5*
9.0	1/ 64	3.413	130*

Table (2): The hemolysis assay results with different sodium chloride concentrations

NaCl %	Hemolytic Unit	Mean of O.D value	Hemolysis percent (100%)
0.5	1/32	2.665	100
1	1/ 64	3.96	152*
2	1/ 64	3.742	143*
3	1/ 32	1.909	70.3*
4	1/ 32	1.811	66*
5	1/ 16	1.399	50*
6	1/ 16	1.202	42*
7	1/ 16	0.981	33*
8	1/ 8	0.733	23*
9	1/4	0.581	17*

O.D: Optical density at absorbance (A: 450 nm) by microtiter plate spectrophotometer, where, mean of optical density for positive control and negative control are (2.508) and (0.145) respectively.

*: Meaning significant differences at ($P \leq 0.05$).

Discussion:

The virulence of *L.monocytogenes* is attributed to Listeriolysin O , this bacteria has ability to survive and multiply inside macrophages. All isolates of *L. monocytogenes* isolated from infections cases produce a hemolysis on blood agar and it has virulent in the mouse model (Rocoutt *et al.*, 1983), these virulence isolates were secreted β-hemolysin (Listeriolysin O). In this study, we explored two experiments for determination of some environmental parameters on activity listeriolysin O on sheep red blood cells suspension (SRBCs). The first experiment by using different levels of pH in trypticase soya broth medium was adjusted by (1M) of NaOH and (1M) HCl. A second experiment using different salt concentration (NaCl). These experiments showed that different levels of environment parameters have an essential effect on the activity of listeriolysin O. Our results demonstrate that high level of pH (Alkaline

pH) in the medium lead to enhance of listeriolysin activity on sheep red blood cells. Whereas, low level of pH (Acidic pH) in a medium which appeared reduction in listeriolysin activity. This evidence consistent with (Datta and Kothary, 1993) who indicated that listeriolysin O production reduced due to a decrease in pH as a result of the growth of Listeria cells in media containing high glucose concentrations. On another hand low level of salt concentration showed increases in listeriolysin O activity but high levels appeared severe reduction in listeriolysin O activity. These results agree with results by (Myers *et al.*, 1993) who indicated that there are significant increases in catalase activity and listeriolysin O activity when wild-type Listeria monocytegenes isolates (10403S) grown in medium containing low levels salt. (Coffey *et al.*, 1996) who study many of environmental parameters, pH, salt concentration and temperature, are effected on the production of another virulence factor like (lecithinase) in *Listeria monocytogenes*. Our result explained that listeriolysin O activity. Therefore, we concluded that environmental parameters like (pH and salt concentration) effect on listeriolysin O production that responsible for the development of listeriosis in human and animals.

References:

Coffey, A., F. M. Rombouts, and T. Abee. 1996. Influence of environmental parameters on phosphatidylcholine phospholipase C production in *Listeria monocytogenes*: A convenient method to differentiate *L. monocytogenes* from other *Listeria* species. Appl. Environ. Microbiol. 62:1252–1256.

Dallmier, A. W., and S. E. Martin. 1990. Catalase, superoxide dismutase, and hemolysin activities and heat susceptibility of Listena monocytogenes after growth in media containing sodium chloride. Appl. Environ. Microbiol. 56:2807-2810.

Datta, A. R., and M. H. Kothary. 1993. Effects of glucose, growth temperature, and pH on listeriolysin O production in *Listeria monocytogenes*. Appl. Environ. Microbiol. 59:3495–3497.

Farber, J. M., and P. I. Peterkin. 1991. Listenra monocytogenes, a food-borne pathogen. Microbiol. Rev. 55:476-511.

Gedde, M. M., D. E. Higgins, L. G. Tilney, and D. A. Portnoy. 2000. Role of listeriolysin O in cell to-cell spread of *Listeria monocytogenes*. Infect. Immun. 68:999–1003.

Goebel, W. and Kuhn, M., Bacterial replication in the host cell cytosol, *Curr. Opin. Microbiol.*, 3, 49, 2000.

Leimeister-Wächter, M., C. Haffner, E. Domann, W. Goebel, and T. Chakraborty. 1990. Identification of a gene that positively regulates listeriolysin, the major virulence factor of *Listeria monocytogenes*. Proc. Natl. Acad. Sci. USA 87:8336–8340.

Myers, E., Dallmier, A. W., and Martin, S. E. (1993). Sodium Chloride, Potassium Chloride, and Virulence in Listeria monocytogenes. Appl. Environ. Microbiol. 59:2082-2086.

Rocourt, J.; Alonso, J.M. and Seelioer, H.P.R. (1983). Virulence comparée des cinq groupes génomiques de *Listeria monocytogenes* (sensu lato). Ann. Microbiol. (Inst. Pasteur) 134:359–364.

Sampathkumar, B., Tsougriani, E., Yu, L.S.L., Khachatourians, G.G., 1998. A quantitative microtiter plate hemolysis assay for *Listeria monocytogenes*. J. Food Safety 18, 197–203.

Vazquez-Boland, J. A.; Kuhn M.; Berche P.; Chakraborty T.; Dominguez-Bernal G.; Goebel W.; Gonzalez-Zorn B.; Wehland J. and Kreft J. (2001). Listeria pathogenesis and molecular virulence determinants. Clin Microbiol Rev 14: 584–640.

تأثير الإجهاد الكيميائي والبيئي على إنتاج الليستيريوليسين O في الليستيريا مونويتوجينس .

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يعتبر اللستيريوليسين او من أهم عوامل الضراوة في جرثومة اللستيريا مونوسايتوجين الذي يتطلب في بقاء الجرثومة داخل خلايا المضيف وبتالي إنشاء الإصابة بمرض اللستيريوسز. في هذه الدراسة قيم تأثير بعض المعايير البيئية التي تتضمن الأس الهيدروجيني وتركيز الملح علي نشاط اللستيريوليسين او لعترة المرضية لجرثومة اللستيريا مونوسايتوجينس في وسط مرق تربتكيز الصويا باستخدام اختبار التحلل الدموي الكمي. حيث أعتبر نشاط اللستيريوليسين او مثالياً في وسط زرعي ذات أس هيدروجيني pH يتراوح مابين (7.5-7.0)، وتركيز الملح (NaCl) (0.5%)، ودرجة حرارة (37ºC)، وبوجود الأوكسجين. أظهرت نتائج اختبار التحلل الدموي بان نشاط اللستيريوليسين او ينخفض في الأس الهيدروجيني الحامضي ، حيث كانت نتيجة التحلل الدموي هي (pH 5.5: 24.5%)، (pH 6.0: 33%)، و(pH 6.5: 77.5%). بالتباين إلى نتائج لتحلل الدموي الكمي في الأس الهيدروجيني القاعدي، حيث أظهرت النتائج بان هنالك تحسين واضح في نشاط اللستيريوليسين او. حيث كانت نتائج التحلل كالأتي (pH 8.0: 122.5%)، (pH 8.5: 150.5%)، و (pH 9.0: 130%). وقد أظهرت هذا النتائج وجود فروقات معنوية بمستوى احتمال ($P\leq 0.05$) مقارنة مع أس الهيدروجين المثالي. إما في الجانب الأخر، فقد أظهرت نتائج التحلل الدموي الكمي مع تراكيز مختلفة من ملح كلوريد الصوديوم (NaCl) وجود زيادة في نشاط اللستيريوليسين او في تراكيز منخفضة من الملح، حيث كانت نتيجة التحلل الدموي هي (152%) في تركيز (1%NaCl) و (143%) في تركيز (2% NaCl). بينما أظهرت نتائج التحلل الدموي انخفاض في نشاط اللستيريوليسين او في مستويات أعلى من تركيز ملح كلوريد الصوديوم ، حيث كانت كالأتي (70.3%) في تركيز (3% NaCl)، (66%) في تركيز (4% NaCl)، (50%) في تركيز (5% NaCl)، (42%) في تركيز (6% NaCl)، (33%) في تركيز (7% NaCl)، (23%) في تركيز (8% NaCl)، (17%) في تركيز (9% NaCl). من تلك النتائج نستنتج بان الأس الهيدروجيني الحامض والقاعدي، وكذلك المستويات المختلفة مت تركيز ملح كلوريد الصوديوم قد يوثر على نشاط اللستيريوليسين او المسؤول عن تطور الإصابة بمرض اللستيريوسس في الإنسان.