Iran J Public Health, Vol. 51, No.12, Dec 2022, pp.2783-2790

Original Article

Evaluation of Chitosan Nanoparticle Antimicrobial Effect on Isolated *Listeria monocytogenes* Bacteria from Pregnant Women and *L. monocytogenes* ATCC 7644

Sara Kazemi Rad [1], ****Mehdi Assmar*** [1], ***Mirsasan Mirpour*** [1], ***Mohamad Reza Razavi*** [2]

1. *Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran*
2. *Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran*

***Corresponding Author:** Email: Mehdiassmar@yahoo.com

(Received 08 Dec 2021; accepted 19 Mar 2022)

Abstract

Background: *Listeria monocytogenes* is a gram positive, facultative intracellular bacteria and it is a causative agent of *listeriosis*. Abortion is one the most important side effect of *listeriosis*. Nano chitosan is widely used as nano-materials considered due to its characteristics such as bactericidal and nontoxicity activity. The aim of this study was isolation of *L. monocytogenes* bacteria from pregnant women vaginal samples and evaluation of chitosan nanoparticles effects against them.

Methods: Overall, 100 vaginal specimens were collected from pregnant women with and without a history of abortion referred to Tehran's Hospitals from Sep 2019 to Jul 2020 with questionnaires. Then, using biochemical methods, *L. monocytogenes* bacteria were isolated and identified. Isolated *L. monocytogenes* strains were confirmed using PCR and evaluation of *prfA* gene, which is the main gene for identification of this bacterium. The effect of chitosan nanoparticles was evaluated in comparison with the antibiotics used based *CSLI* guideline on isolated bacteria by well diffusion method. *MIC* and *MBC* were determined for nanoparticle at the end.

Results: Five strains of *L. monocytogenes* that were confirmed by PCR method. Moreover, a statistically significant relationship was observed between the isolated strains and the samples taken from women with a history of abortion. MIC and MBC for *L. monocytogenes* ATCC 7644 were 156.25 and 312.5 µg/ml and for 5 isolated strains were 78.12 and 158.25 µg/ml, respectively.

Conclusion: *L. monocytogenes* could be a causative agent of abortion in pregnant women. Concerning the results, Nano chitosan has acceptable antibacterial activity against *L. monocytogenes*.

Keywords: *Listeria monocytogenes*; Abortion; *prfA* protein; Chitosan nanoparticles

Introduction

Listeria is Gram-positive bacteria, rod-shaped, facultative anaerobic, and non-spore forming (1). The genus *Listeria* contains eight species including *Listeria monocytogenes,* which is pathogenic for humans and animals, and the species *L. ivanovo,* which is often pathogenic for animals but rarely for humans. *L. monocytogenes* causes a disease calls *listeriosis* that affects humans and animals (*zoonosis*). *L. monocytogenes* is bacterial food borne infections, can result in abortion and diseases as se-

vere as gastroenteritis, encephalomeningitis and septicemia (2, 3). Isolation of this bacterium from infected specimens with different species requires the use of enriched media and conventional biochemical methods.

Since their discovery, antimicrobial drugs have proved remarkably effective for the control of bacterial infections. However, bacterial pathogens were unlikely to surrender unconditionally, because some pathogens rapidly became resistant to many of the first effective drugs. Nanotechnology has provided promising approaches to solve this issue. It offers a great chance to treat drug-resistant microbial infections. A nanoparticle is a small particle that ranges between 1 to 100 nanometers in size. It can exhibit significantly different physical and chemical properties to larger material. They can constructed from different biodegradable material like natural or synthetic polymers, phospholipids or lipid (4). Nanoparticles have many advantages such as increased bioavailability, delivery of specific drug to a specific location, continuous drug delivery and increased patient compliance due to frequent reduction of drug dose (5). Artificial nanoparticles can be created from any solid or liquid material, including metals, dielectrics, and semiconductors. They may be internally homogeneous or heterogeneous with a Core–shell structure. There are several methods for creating nanoparticles, including gas condensation, attrition, chemical precipitation, ion implantation, pyrolysis and hydrothermal synthesis (6).

Chitosan nanoparticles is produced by the partial alkaline nitrogenation of N-chitin, which is commercially extracted from shrimp and crab shells. It is also found in nature, in the cuticle of insects and the cell wall of fungi (6, 7). In recent years, chitosan and its derivatives have attracted much attention as antimicrobial agents against bacteria, fungi and viruses (8-10). Due to its biodegradability, biocompatibility and non-toxicity, it uses as a new biomaterial for food processing (11), pharmaceutical compounds (6, 12), medicine (13, 14), agriculture (15), and industry (16).

The aim of this study was isolation of *L.monocytogenes* bacteria from pregnant women vaginal samples and evaluation of chitosan nanoparticles effects against them.

Materials and Methods

Sampling and primary identification

Overall, 100 vaginal samples were collected from pregnant women with and without a history of abortion referred to Tehran's Hospitals from Sep 2019 to Jul 2020 with questionnaires.

The initial proposal of the work was approved by Ethics Committee of the Azad University of Lahijan, Iran, and necessary permission was received for the work.

Vaginal swabs were put in 10mL Trypticase Soy Broth with yeast extract (TSBYE), and incubated at 4 °C for one week. Then, they were inoculated on blood agar (Merck, Germany) and PALCAM Listeria agar (Merck, Germany) using streaking culture and incubated at 37 °C for 24 to 48 h. Bacteria with Yellow small colonies with β-hemolysis on blood agar and Green shiny colonies with diffused black shadows around them on PALCAM listeria agar are probably Listeria. Isolated bacteria were identified using biochemical tests (17,18).

Molecular Identification

Isolated *Listeria monocytogenes* strains using biochemical tests were confirmed using PCR and evaluation of *prfA* gene, which is one of the main genes for identification of this bacterium. DNA extraction and PCR set up were done in standard procedure using guide. The primers and PCR program is in Table 1 (19-21).

Chitosan preparation

Chitosan nanoparticles were used in this study (22). The particle diameter is 22-80 nm and its purity is 99%. A solution of 10 mg / ml in 2% acetic acid was prepared and sterilized at 121 °C and a pressure of 1.5 atmospheres for 20 minutes. The solution pH was adjusted on 5 and stored at 4 °C for later use (23).

Table 1: Primers and PCR programming

Primers	*prfA F: GAAGTCATTAGCGAGCAGGC* *prfA R: ATGCCACTTGAATATCCTAACTCC*
Program	95 °C for 10 min and 30 cycles of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, followed by a final extension step at 72 °C for 5 min.

Antimicrobial activity

Well diffusion method was used to evaluate the antimicrobial activity of chitosan nanoparticles. In this method, after making a hole in Müller-Hinton agar medium previously cultured with isolated *L. monocytogenes* and *L. monocytogenes* ATCC 7644, 20 mL of different concentrations of chitosan nanoparticles (4.88 to 5000 µg/ml) were poured in wells. The plates were incubated at 37 °C for 24 h. The inhibition zone for the wells was measured. The effect of common antibiotics based on CSLI on clinical and standard strains was also investigated.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC were done using the Macrodilution test. The minimum inhibitory concentration method was used to determine the susceptibility of strains to chitosan nanoparticles. To determine the minimum inhibitory concentration, a suspension of fresh bacterial culture with 0.5 McFarland (1.5×10^8 Cells/ml) was prepared. Then, different concentrations of nanoparticles (4.88 to 5000 µg/ml) by Trypticase soy broth (1 mL) were done. 100 microliters of microbial suspension was added to all tubes and they were incubated for 24 h at 37 °C. The turbidity of the tubes is checked and the concentration of first transparent tube is the MIC. The next transparent tubes are then cultured in Müller-Hinton agar. The first concentration at which 99.99 microbes were killed is MBC (24).

Statistical analysis

All data were compiled, and analysis was done using SPSS version 26 (IBM Corp., Armonk, NY, USA). *P*-value less than 0.05 were considered as significant.

Results

Sampling, Isolation and Identification

Overall, 100 women were included in the study. Following culture and differential tests (Table 2), 5 *L. monocytogenes* were isolated and identificated from vaginal specimens. Demographic and clinical features of the patients are given in Table 3.

Table 2: Biochemical tests to differentiate *Listeria* species

Species	***Phospholipase c***	***Hemolysis***	***Production of acid from***			***CAMP test (S.aureus)***
			D-Mannitol	L-Rhmnose	D-Xylose	
L. monocytogenes	+	+	-	+	-	+
L. innocua	-	-	-	V	-	-
L. ivanovii	+	++	-	-	+	-
L. seeligeri	-	(+)	-	-	+	(+)
L. welshimeri	-	-	-	V	+	-
L. grayi subsp. Grayi	-	-	+	-	-	-
L. grayi subsp. Murrayi	-	-	-	V	-	-

V: variable, (+): weak reaction, ++: strong positive reaction, +: >90 % positive reactions, -: negative reaction

Table 3: Frequency of *Listeria monocytogenes* based on Research Variables

Variables	*Positive*	*Negative*	*Total*
Age			
15-25 yr	0	10	10
26-35 yr	3	59	62
36-45 yr	2	22	24
Higher than 45	0	4	4
Total	5	95	100
Education			
Low level of education	1	8	9
High school diploma	3	25	28
Higher than diploma	1	62	63
Total	5	95	100
Job			
Housewife	3	46	49
Employed	2	49	51
Total	5	95	100
Abortion precedence			
Abortion precedence	3	8	11
Non-Abortion precedence	2	87	89
Total	5	95	100
Number of Pregnancy			
First	0	49	49
Second	3	30	33
Third	2	12	14
More than third	0	4	4
Total	5	95	100
History of preterm delivery			
History of preterm delivery	1	20	21
No history of preterm delivery	4	75	79
Total	5	95	100
Taking antibiotics			
Taking antibiotics	4	35	39
Do not take antibiotics	1	60	61
Total	5	95	100

Nine patients (9%) were low educated, 28 (28%) had a high school diploma and 63 (63%) had a college degree. High school graduates and low-educated patients had the highest incidence of *L. monocytogenes* compared with higher educated ones (3,1,1 patients). Therefore, there was a significant association between positive patients and control samples regarding education.

Of 100 patients, 11 (11%) had abortion before and 89 (89%) did not have any abortion history. 3 patients (60%) of 5 positive patients had abortion precedent. There was a significant association between patients with abortion precedence and with no abortion precedence.

Results indicates that of 5 patients positive for *L. monocytogenes*, 3 (60%) and 2 (40%) were housewives and employed women. Hence, there was no significant association between the housewives and the employed women ($P = 0.9$).

Antimicrobial activity

Antimicrobial effect of concentrations of 4.88 to 39.06 µg/ml chitosan nanoparticles on clinical strains of *L. monocytogenes* isolated from pregnant women and 4.88 to 78.12 µg/ml on standard *L. monocytogenes* ATCC 7644 strain showed that the concentrations had no effect on bacteria ($P>0.05$) (Table 4). The results of Antibiogram tests using the CSLI reference in disc diffusion method are also shown in Table 5.

Based on the results, MIC and MBC for chitosan nanoparticles were different in clinical and standard strains (Table 6).

Table 4: Diameter of chitosan nanoparticle growth inhibition zone on clinical and standard strains in well diffusion method (mm)

Bacteria / ***Chitosan Nanoparticle concentration (µg/mL)***	***Listeria monocytogens A***	***Listeria monocytogens B***	***Listeria monocytogens C***	***Listeria monocytogens D***	***Listeria monocytogens E***	***Listeria monocytogens ATCC 7644***
4.88	-	-	-	-	-	-
9.76	-	-	-	-	-	-
19.53	-	-	-	-	-	-
39.06	-	-	-	-	-	-
78.12	10	9	8	7	7	-
156.25	13.6	12	12.3	13	11	11
312.5	17	15	14	15	13.6	13.6
625	20	18	19	18.3	17	17.6
1250	23.3	22	23.6	20	21	20.3
2500	27	26	27.3	24	25.3	23.6
5000	31	29	30	28	27	28

Table 5: Diameter of Antibiotics growth inhibition zone on clinical and standard strains in disc diffusion method (mm)

Bacteria / ***Antibiotics***	***Listeria monocytogens A***	***Listeria monocytogens B***	***Listeria monocytogens C***	***Listeria monocytogens D***	***Listeria monocytogens E***	***Listeria monocytogens ATCC 7644***
Gentamycin	20	17.6	19	23	24.6	21
Ampicillin	25	19	17.3	20.3	21	23
Tetracycline	9.6	13	15	16	17.6	11.3
Cotrimoxazole	20	19	20.6	17.3	24.6	25
Ciprofloxacin	21	23.6	21.3	25	21	21.3
Erythromycin	23	26	12	25	13	23
Penicillin	24	0	21	0	21	24.6
Meropenem	25	24	20.6	19	24.6	26
Nalidixic acid	13	19	15.6	20.6	13	21
Chloramphenicol	0	18	12	0	20	0
Ceftazidime	20	13	18	13.6	19	18

Table 6: MIC and MBC for Chitosan nanoparticles

Strains ***Kind of Inhibition***	***Listeria monocyto-gens*** ***ATCC 7644***	***Listeria monocyto-gens*** ***A,B, C, D, E strains***
MIC (µg/mL)	156.25	78.12
MBC (µg/mL)	312.5	156.25

Discussion

Approximately one-third of the prevalence of human *listeriosis* is related to pregnancy and causes miscarriage, especially in the second or third trimester. In the present study, five strains of *L. monocytogenes* were isolated and identified from pregnant women using culture techniques and PCR confirmation. The results and statistical evaluations of the data have shown that *L. monocytogenes* can cause abortion in pregnant women. Many factors are involved in manifestations of *L. monocytogenes* virulence. Seven and 36 strains of *L. monocytogenes* were isolated from 100 vaginal specimens in Iran using culture and PCR methods, respectively. PCR was faster, more specific and more sensitive than culture to detect *L. monocytogenes* in vaginal swabs (25).

In India, 4 *L. monocytogenes* were isolated from 305 different samples of urine, blood, feces, placenta and vaginal swabs collected from 61 aborted women (26).

Out of 311 samples collected from 107 pregnant women (190 samples from pregnant women with a history of abortion and 120 cases without it), *L. monocytogenes* was detected in 11 cases (10.28%). The highest frequency was in the age group of 26 to 30 yr. The mean age of participants was 26.7 yr and approximately 64% of positive samples were taken from the cervix (27). The prevalence of *Listeria monocytogenes* in pregnant women with a history of abortion is higher than pregnant women without a history of abortion and these results are in accordance with the present study.

Overall, 71 samples of seafood were examined. Twenty cases (28.2%) of *L. monocytogenes* were identified, of which 15 cases (75%) were confirmed as virulent strains. In addition, from 50 human fecal samples, only 1 case (2%) was identified with virulent *L. monocytogenes* (28).

In China, out of 548 samples of pre-prepared foods on the market, 32 strains (5.8%) were isolated. The prevalence of *L. monocytogenes* in pre-prepared foods on the market is significantly higher than food cooked in restaurants and hotels (29).

In this study, the antibacterial activity of chitosan nanoparticles in culture medium was investigated. The antibacterial effect of nanoparticles on reducing cell number and survival of *L. monocytogenes* was astonishing.

Bacterial damage caused by acidic and water-soluble chitosan concentrations. The highest inhibitory effect was observed in 10% acid-soluble chitosan, while this effect was less in water-soluble chitosan. Chitosan had the greatest inhibitory effect on *L. monocytogenes* compared to common antibiotics, and in some cases, acid-soluble chitosan, like common antibiotics, prevented the growth of bacteria. In other words, bacteria resistant to common antibiotics were sensitive to chitosan (30).

With a wide range of antimicrobial activities, chitosan exhibits different inhibitory effects on gram-positive and gram-negative bacteria. Antibacterial activity has complex stages in gram-positive and gram-negative bacteria based on cell surface characteristics. Chitosan has been reported to have a stronger antibacterial effect on gram-positive bacteria than gram-negative (31). This may be due to the barrier of the outer membrane in gram-negative bacteria (32). The appropriate effect of chitosan nanoparticles on *L. monocytogenes* (gram-positive bacteria) in this study confirms this (33).

Conclusion

L. monocytogenes is regarded a causative agent of abortion in pregnant women. We showed that Nano chitosan has acceptable antibacterial activity against *L. monocytogenes*.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

The authors acknowledge the cooperation and assistance of Day's hospital laboratory (Tehran, Iran).

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Vazquez-Boland J, Kuhn M, Berche P, et al (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clin Microbiol Rev*, 14:584–640.
2. Pust S, Morrison H, Wehland J, Sechi AS, Herrlich P (2005). *Listeria monocytogenes* exploits ERM protein functions to efficiently spread from cell to cell. *EMBO J*, 24(6):1287–300.
3. Neuhaus K, Satorhelyi P, Schauer K, Scherer S, Fuchs TM (2013). Acid shock of *Listeria monocytogenes* at low environmental temperatures induces prfA, epithelial cell invasion, and lethality towards Caenorhabditis elegans. *BMC Genomics*, 14:285.
4. Kayser O, Lemke A, Hernandez-Trejo N (2005). The impact of nanobiotechnology on the development of new drug delivery systems. *Curr Pharm Biotechnol*, 6(1):3–5.
5. Chen DB, Tian ZY, Wang LL, Qiang Z (2001). In vitro and in vivo study of two types of long-circulating solid lipid nanoparticles containing paclitaxel. *Chem Pharm Bull (Tokyo),* 49(11):1444–7.
6. Singla A. K, Chawla M (2001). Chitosan: some pharmaceutical and biological aspects—an update. *J Pharm Pharmacol*, 53:1047–1067.
7. Tharanathan R, Kittur F(2003). Chitin-the undisputed biomolecule of great potential. *Crit Rev Food Sci Nutr*, 43:61-87.
8. Chirkov S (2002). The antiviral activity of chitosan. Appl. *Biochem Microbiol*, 38:1–8.
9. Muzzarelli R, Tarsi R, Filippini O, Giovanetti E, Biagini G, Varaldo E (1990). Antimicrobial properties of N-carboxybutyl chitosan. *Antimicrob Agents Chemother*, 34:2019-2023.
10. Rabea E, Badawy M, Stevens C, Smagghe G, Steurbaut W (2003). Chitosan as antimicrobial agent. Applications and mode of action. *Biomacromolecules*, 4:1457–1465.
11. Rhoades J, Roller S (2000). Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. *Appl Environ Microbiol*, 66:80-86.
12. Illum L (1998). Chitosan and its use as a pharmaceutical excipient. *Pharm Res*, 15:1326–1331.
13. Ueno H, Mori T, Fujinaga T (2001). Topical formulations and wound healing applications of chitosan. *Adv Drug Deliv Rev*, 52:105–115.
14. Ylitalo R, Lehtinen S, Wuolijoki E, Ylitalo P, Lehtima¨ki T(2002). Cholesterol-lowering properties and safety of chitosan. *Arzneimittelfors-chung*, 52:1–7.
15. Doares, S, Syrovets T, Weiler E, Ryan C (1995). Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. *Proc Natl Acad Sci USA*, 92:4095–4098.
16. Babel S, Kurniawan T (2003). Low-cost adsorbents for heavy metals uptake from contaminated water. *J Hazard Mater*, 97:219–243.
17. Patel J, Beuchat, L (1995). Enrichment in Fraser broth supplemented with catalase or oxyrase, combined with the microcolony immunoblot technique, for detecting heat-injured Listeria monocytogenes in foods. *Int J Food Microbiol*, 26 : 165–176.
18. Donnelly C (2002). Detection and isolation of *Listeria monocytogenes* from food samples: impli-

cations of sublethal injury. *J AOAC Int*, 85: 495–500.
19. Sanger F, Nicklen S, Coulson A (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA*, 74: 5463–5467.
20. Rasmussen O, Skouboe P, Dons L, Rossen L, Olsen J (1995). *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. *Microbiology*, 141: 2053–2061.
21. Cai S, Kabuki D, Kuaye A, et al (2002). Rational design of DNA sequence-based strategies for subtyping *Listeria monocytogenes*. *J Clin Microbiol*, 40: 3319–3325.
22. Sogol A, Mehdi A, Mirsasan M (2020). Effect of Chitosan Nanoparticle from Penaeus semisulcatus Shrimp on *Salmonella typhi* and *Listeria monocytogenes*. *Iran J Public Health*, 49 (2): 369-376.
23. El-Hefian E, Elgannoudi E, Mainal A, Yahaya A (2010). Characterization of chitosan in acetic acid. Rheological and thermal studies. *Turk J Chem*, 34 (2): 47 – 56.
24. Ketabchi M, Iessazadeh K, Massiha A (2017). Evaluate the inhibitory activity of ZnO nanoparticles against standard strains and isolates of *Staphylococcus aureus* and *Escherichia coli* isolated from food samples. JFM, 4(1):63-74.
25. Morteza S, Mahdi F (2009). Isolation and identification of *Listeria monocytogenes* in vaginal samples using PCR method. *Modares J Med Sci*, 12:51–58.
26. Kaur S, Malik SV, Vaidya VM, Barbuddhe SB (2007). *Listeria monocyt ogenesin* spontaneous abortions in humans and its detection by multiplex PCR. *J Appl Microbiol*, 103(5):1889–96.
27. Jahangirsisakht A, Kargar M, Mirzaee A, et al (2013). Assessing *Listeria monocytogenes* hly A gene in pregnant women with spontaneous abortion using PCR method in Yasuj, southwest of Iran. *Afr J Microbiol Res*, 7(33):4257–60.
28. Ahmed HA, Hussein MA, El-Ashram AM (2013). Seafood a potential source of some zoonotic bacteria in Zagazig, Egypt, with the mo-lecular detection of *Listeria monocytogenes* virulence genes. *Vet Ital*, 49(3):299–308.
29. Chao G, Deng Y, Zhou X, et al (2006). Prevalence of *Listeria monocytogenes* in delicatessen food products in China. *Food Control*, 17(12):971-4.
30. Mitra S, Gaur U, Ghosh PC, Maitra AN (2001). Tumour targeted delivery of encapsulated dextran–doxorubicin conjugate using chitosan nanoparticles as carrier. *J Control Release*, 74: 317-323.
31. Chen F, Shi Z, Neoh KG, Kang ET (2009). Antioxidant and antibacterial activities of eugenol and carvacrolgrafted chitosan nanoparticles. *Biotechnol Bioeng*, 104: 30-39.
32. Xing K, Chen XG, Li YY, et al (2008). Antibacterial activity of oleoyl-chitosan nanoparticles: A novel antibacterial dispersion system. *Carbohydrate Polymers*, 74: 114-120.
33. Shrestha A, Hamblin MR, Kishen A (2014). Photoactivated rose bengal functionalized chitosan nanoparticles produce antibacterial/biofilm activity and stabilize dentincollagen. *Nanomedicine*, 10: 491-501.