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Effects of Chitosan Nanoparticles on Mice Infected with *Listeria monocytogenes*

Sara Kazemi Rad [1], Mirsasan Mirpour [2*], Mehdi Assmar [3], Mohamad Reza Razavi[4]

[1]Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran
[2]Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran
[3]Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran
[4]Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran

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Abstract

Listeria monocytogenes is the cause of listeriosis, which has many complications, especially in pregnant women. Due to the antibiotic resistance of this bacterium, many attempts have been made to introduce different medicinal compounds, including nanoparticles based on biological compounds. This research aimed to examine the mechanism of the effect of chitosan nanoparticles on *L. monocytogenes* in vivo (in the body of a living organism). The standard strain of *L. monocytogenes* (ATCC 7644) was prepared and analyzed in the Day Hospital Laboratory (Iran). The bacteria were examined based on biochemical tests. Then, the antibacterial activity of concentrations of 4.88 to 5000 µg/mL of chitosan nanoparticles against *L. monocytogenes* standard (ATCC 7644) was calculated with the investigated methods and the lowest inhibitory and bactericidal concentrations (MIC and MBC, respectively). The effects of different nanoparticle concentrations and ampicillin in mice infected with bacteria were also investigated. In infected mice, the therapeutic effect increased with increasing the nanoparticle concentration, and the concentration of 156.25 µg/mL was the most effective compared to other treatments. Also, ampicillin (10 µg) and chitosan nanoparticles with a concentration of 39.06 µg/mL had almost the same therapeutic effect. With the timely identification of listeria contamination in pregnant women and the proper use of chitosan nanoparticles instead of common drugs, a new solution can be found for the treatment of listeriosis.

Key words: Listeriosis, nanoparticles, antibacterial properties, Balb/c mice

*Corresponding Author: E-mail: sassan47@gmail.com

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1. Introduction

Listeria monocytogenes coccobacillus is Gram-positive, rod-shaped, short, regular, without spores, catalase-positive, and oxidase-negative; it can survive in the range of pH = 4.4-9.6 and is an opportunistic intracellular pathogen that can grow at 0 to 45 °C. Among Listeria species, *L. monocytogenes* is pathogenic for humans and is very important because of its role in human infection caused by food around the world (Manso, 2019, Mook, 2011). This bacterium causes listeriosis, which is an infectious disease. Listeriosis is one of the common but little-known diseases of the present era that affects humans and domestic and wild animals. It is mainly transmitted through food contaminated with *L. monocytogenes* (Zhang, 2021, Pizarro-Cerdá, 2019). *L. monocytogenes* can cause miscarriage, stillbirth, meningitis, and encephalitis in immunocompromised people and people with underlying diseases. In general, dairy products, unpasteurized milk, soft and semi-soft cheese, raw meat, vegetables, and fish are the main sources of listeriosis (Altuntas, 2012, Haubert, 2016 , Zarei, 2013). In 1932, the first study of the effect of listeriosis on pregnant animals was carried out, and during an epidemic, a Gram-positive bacillus was isolated, which resulted in abortion when entered into a vein or vagina; the microbe can be found in the fetal viscera, vaginal secretions, and uterine wall.. Listeria can stay in the reproductive organs for a relatively long time, cause frequent miscarriages, and even lead to infertility and keep its listeriosis hidden in all these stages. Resistance to human pathogens is a big challenge in the field of medicine. These antibiotic resistances and the continuous and indiscriminate use of chemical medicinal compounds cause the serious phenomenon of resistancein microorganisms (Mota, 2020, Caruso, 2020, Yang, 2020), and for this reason, many studies have been done in connection with nanoparticles all over the world. One of these nanoparticles is called chitosan, which is one of the most important derivatives of chitin. Chitin is a mucopolysaccharide that has a structural role, is abundantly found in the exoskeletons of arthropods such as shrimp, and crabs (Steckel, 2004). One of the most important derivatives of chitin is a compound called chitosan, which, unlike chemical polymer compounds, is nontoxic and decomposes in nature. Chitosan is a biopolymer derived from the removal of acetyl from chitin (Dutta, 2002, Dutta, 2004). The conversion of chitin to chitosan can be done using sodium or potassium hydroxide solutions (40-50%) at a temperature of 100 °C or higher by removing parts or all of the acetyl groups from the polymer. Ampicillin, in combination with an aminoglycoside such as gentamicin or streptomycin, is the first choice for the treatment of listeria. Ampicillin, penicillin, or rifampin alone or in combination with gentamicin are standard antibiotic treatments for listeriosis (Altuntas , 2012). The classification of the use of this antibiotic in pregnancy is group B, and it should also be used with caution during breastfeeding (Haginaka,1987 , Jung, 2006, Jung, 2012).

Materials and Methods

Bacterial Strain and Culture Conditions

The standard strain of *L. monocytogenes* (7644 ATCC) was purchased in the lyophilized form from the microbial collection of Pars Bioproduct Research and Production Center, and the vial was broken under sterile conditions. For activation of strains, the contents of the vial were placed in screw-capped tubes, each containing 10 mL. A liter of TSB medium was transferred to activate the bacteria, and the bacteria were incubated for 24 hours at a temperature of 37 °C. After the mentioned period, the activated bacteria were transferred to the brain heart infusion (BHI) culture medium and kept in a greenhouse for 24 hours at a temperature of 37 °C and then at 4 °C .Finally, diagnostic biochemical tests were performed to confirm the strains, which include: Gram staining, catalase, oxidase, Sulfur, Indole, Motility (SIM), hemolysis, Christie-Atkins-Munch-Petersen(CAMP) , methyl red-Voges Proskauer (MR-VP), acid production from rhamnose, dextrose, xylose, triple sugar iron (TSI), and hydrolysis of bile esculin. A suspension of the standard strain was prepared in tryptic soy broth (TSB) medium, mixed with sterile glycerin at a ratio of 5 to 1, poured into

sterile microtubes, and stored at -20 °C.

Chitosan Nanoparticle Preparation and Characterization

In this study, chitosan nanoparticles (CNPs) produced by Aleboyeh et al. were utilized (Alebouyeh, 2020). The physicochemical characteristics of CNPs were measured by zeta potential , particle size and particle dispersity index (PDI) by dynamic light scattering method using Zetasizer (Malvern, Nano ZS, England). The CNPs had a diameter ranging from 22 to 80 nm and a purity of 99%. The polydispersity index (PDI) was determined to be 0.229. Furthermore, the zeta potential of the CNPs was measured to be -19.5 mV.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the CNPs were determined by the macrodilution method. The concentrations of 4.88 to 5000 µg/mL of CNPs were prepared. Then, 100 microliters of each of the microbial suspensions with a turbidity equal to half McFarland was added to each of the tubes. Next to the test tubes, a positive control tube containing bacterial suspension without CNPs and a negative control containing the TSB culture medium without bacteria were considered. The prepared suspensions were incubated next to the control samples at 37 °C for 24 hours in a shaker incubator at 2000 rpm.

The turbidity of the contents of each test tube was checked. The lowest concentration of the nanoparticle suspension that did not show turbidity in the tube was determined as the MIC of the nanoparticle. Next, 100 microliters of the Mueller-Hinton agar culture medium were cultured in tubes that had no growth and no turbidity and were incubated for 24 hours at 37 °C. After this time, each plate in which the bacteria did not grow was declared as MBC. All the experiments were performed in triplicate (Katabchi, 2017, Kazemi Rad , 2022).

The antibiotic effect of ampicillin on the standard strain

We performed all the steps mentioned for CNPs for ampicillin to compare the performance of the nanoparticle and antibiotic. Dilution of ampicillin was done with distilled water with pH=5.

Mice

Seventy female and 14 male BALB/c mice were purchased from the Razi Institute (Karaj, Iran). All the mice were 21 days old, and their weight was between 18 and 22 grams. The mice were kept in a room with a temperature of 24 ± 2 °C, a 12-hour light/dark cycle, and relative humidity of 40% according to the instructions of the Ethics Committee.

The side effects of the substances used on mice

Four groups of 5 healthy female mice were examined in separate cages. The first group was given 0.2 mL of the TSB medium. The second group was given CNPs with the obtained MIC concentration. The third group was given distilled water with pH=5, which was injected subcutaneously. The fourth group was not injected with any substance and was considered the control group. Then, 1 male mouse was placed inside each cage, and the results were analyzed 10 to 14 days later.

The method of contamination and treatment of mice

The rest of the female mice were divided into 5 groups of 10, and each group was placed in separate cages. Then, 0.2 mL of *L. monocytogenes* bacterium suspension with a concentration of 0.5 McFarland was injected subcutaneously. After 48-72 h, to make sure that female mice were infected, we cut their tail area randomly with scissors in sterile conditions and cultured a drop of blood from the tail area; we also prepared a slide, cultured it in blood agar culture medium, and used it to check the presence of L. monocytogenes. After making sure that the mice were infected, we placed 2 male mice in each cage and waited 10 days for mating to take place (the male mice remained inside the cages until the end of the research); then, we started treatment. To the first group, which is the control group for therapeutic effects, we injected distilled water with pH = 5; to the second group, ampicillin was injected with determined MIC concentration; to the third group, we injected the nanoparticle with pre-MIC concentration; to the fourth group, the nanoparticle MIC concentration was injected; and to

the fifth group, post-MIC concentration (MBC) of CNPs was injected every 8 hours at 0.2 mL. We recorded the results of this treatment process, including fertility or infertility, abortion, physical disabilities in newborns, and maternal and neonatal deaths after 3 weeks. It should be noted that ketamine (100 mg/kg) and xylazine (10 mg/kg) (intraperitoneal injection) were used as sedatives, and 2% lidocaine (spray at the injection site) was used as an analgesic.

Daily care and observations were performed to record the clinical signs and the mortality rate of the mice during the entire treatment period.

Statistical analysis

Data analysis was performed using SPSS v. 26 (IBM Corp., Armonk, NY, USA). P-values less than 0.05 were considered statistically significant.

Results

Antimicrobial activity

The findings of this research show that CNPs and ampicillin have a concentration of 5000 µg/mL compared to the standard strain. In the case of the standard strain, CNPs at concentrations of 78.12 to 4.88 µg/mL did not have any inhibitory effect on the tested bacterial strains. Also, the antibiotic ampicillin had no inhibitory effect at the concentrations of 156.25 to 4.88 µg/mL for the standard strain. Distilled water with pH=5 as a control had no growth halo. The MIC and MBC values of CNPs were 156.25 and 312.5 µg/mL, respectively, for the standard strain and 312.5 and 625 µg/mL for the antibiotic ampicillin, respectively.

Effects of CNPs on infected mice

Control mice had normal reproduction and did not show any problems. In the study conducted on healthy female BALB/C mice, opposite results were obtained. Injection of TSB culture medium, CNPs with MIC concentration (78.12 µg/mL), and distilled water with pH = 5 in groups of 5 female BALB/c mice separately had no side effects compared to the control group in biological activities (fever, decreased feeding, cornering, body hair changes, including color change and spiking, pregnancy, abortion, infant disability, infant mortality, and maternal mortality), and all the biological activities of the mice were completely normal.

In this research, the injection of distilled water with pH = 5 to a group of 10 BALB/c mice infected with *L. monocytogenes* bacteria (group A) was considered as a control, and the results were obtained as follows: Out of 10 mice studied in this group, 70% had fever, 80% were withdrawn, 80% had changes in body hair, and 20% became pregnant; among these pregnant mice, 50% suffered abortion, and maternal mortality was not reported.

In groups of 10 infected female mice treated with ampicillin (156.25 µg/mL) (group B), 20% of the mice had a fever, 30% had decreased feeding, 30% were withdrawn, and 30% showed changes in the appearance of body hair, and 70% of them became pregnant. Among the pregnant mice, 25.8% had abortions, but the others were without clinical symptoms (P-value <0.05 and significant).

In this study, different concentrations of CNPs (39.02-12.78-156.78-25.02 µg/mL) were injected into 3 groups of 10 infected mice separately. The effect of the concentration of 39.02 µg/mL nanoparticles on infected mice (group C) is reported: 20% of the mice had a fever, 30% were isolated, 30% had reduced feeding, 30% showed changes in body hair, and 70% were pregnant. Among the pregnant mice of this group, a 14.3% abortion was seen, and the rest of the pregnant mice had no special complications (P-value<0.05).

The effect of the injection of 12.78 µg/mL nanoparticles on infected mice (group D) was observed as follows: 10% of mice had a fever, 20% had decreased feeding, 20% became withdrawn, 20% showed changes in body hair, and 70% reported pregnancy. Among the pregnant mice of this group, a 14.3% abortion was observed, and other pregnant mice had no symptoms (P-value <0.05 and statistically significant).

In this research, injecting a concentration of 156.25 µg/mL into infected female rats (group E) resulted in the following results: 10% reduction in feeding, 10% withdrawal, 10% changes in appearance (body hair), and 80% pregnancy, but the mice did not develop fever. Among the pregnant rats examined in this group, no complications were reported (P-value<0.05) (Fig1).

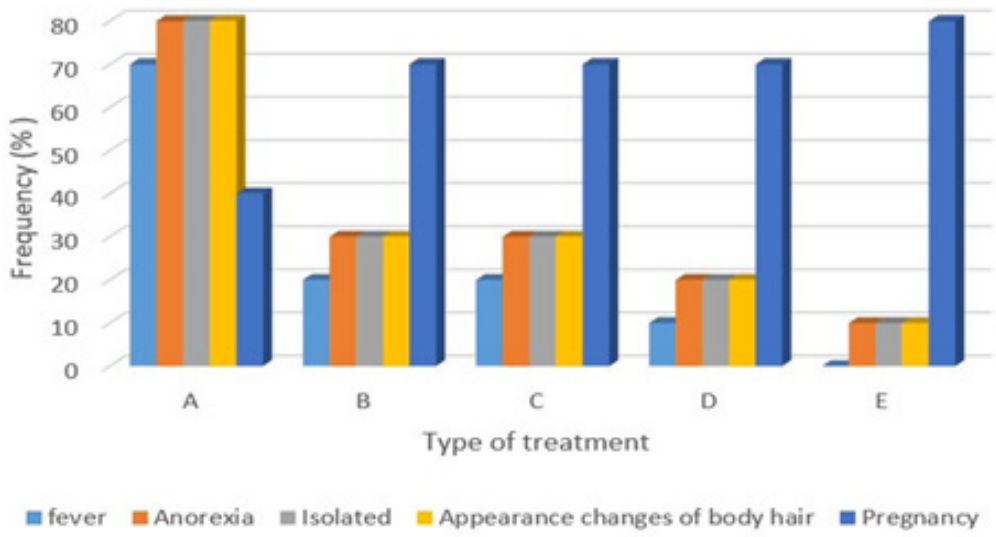

Figure1- Investigating the therapeutic effect of different concentrations of chitosan nanoparticles compared to the antibiotic ampicillin in the treatment of Balb/c mice suffering from listeriosis . A: treatment with distilled water with pH 5 (control), B: treatment with the antibiotic ampicillin (156.25 µg/mL), C: treatment with a concentration of 39.06 µg/mL chitosan nanoparticles, D: treatment with a concentration of 78.12 µg/mL chitosan nanoparticles, E: treatment with a concentration of 156.25 µg/mL chitosan nanoparticles.

Discussion

L. monocytogenes is an opportunistic pathogen and the cause of listeriosis with a wide clinical spectrum. Listeriosis is not a reportable disease in Iran's health and safety system, so the actual prevalence of *L. monocytogenes* in Iran is unknown. Listeriosis is a serious and invasive disease that enters the human body through inhalation, eating contaminated food, using animal products, and direct contact. Among Listeria species, only *L. monocytogenes* is pathogenic for humans. This bacterium is one of the few microorganisms that can pass through the placenta (Vazco-Boland, 2001).

It has been reported that chitosan generally has a stronger bactericidal effect on Gram-positive bacteria than Gram-negative bacteria (Jeon, 2001). This is probably due to the outer membrane barrier of Gram-negative bacteria (Zhang, 2021). In the research conducted, *L. monocytogenes* is a Gram-positive bacterium, and CNP has shown a significant inhibitory effect on this bacterium.

The low solubility of chitin is considered the most important factor limiting the use of this biopolymer. Despite this limitation, so far, many applications of chitin and its derivatives have been reported. When chitin is soluble in acidic aqueous solvents whose pH is less than 6, it is called chitosan.

Chitosan has important functional groups, such as amine groups, at the second carbon position and primary and secondary hydroxyl groups at the third and sixth carbon positions. Chitosan has antimicrobial properties that are created through interactions between the positive charge of the amine group and the negative charge on the cell wall of the microorganism. Among its prominent features are its biodegradability, high biocompatibility, and nontoxicity. According to the literature, antimicrobial activity is highly dependent on the degree of deacetylation and molecular weight, and it can also have different effects according to the bacterial strain (Aranaz, 2009 , Pillai, 2009 , Chen, 1998 , Feng, 2011, Chavez, 2011).

In fact, the antibacterial property of CNP depends on the protonation of amino groups, which can react with the negative charge of the cell surface and lead to the destruction of bacterial cells (Wei 2009). Perhaps in the present study, CNPs have a high positive charge density and have a good inhibitory effect on clinical and standard strains of L. monocytogenes; also, in this re-

search, we dissolved chitosan at pH 5 and performed the other tests.

Radam et al. (2020) aimed to investigate the effect of dietary chitosan supplementation on the experimental infection of mice by L. monocytogenes. The results showed the isolation of bacteria mainly from the brain, spleen, and liver of positive control groups, while it showed slight growth in the other treated groups. In our research, symptoms decreased after treatment with chitosan nanoparticles (Radam, 2020).

In the research conducted by Ma et al., it was shown that the antimicrobial properties of CNPs increase with the increase in the concentration of these particles (Ma , 2010), which was also obtained in our research and the lack of growth halo. Bacteria, due to different concentrations of these nanoparticles, showed that the greatest inhibitory effect in chitosan with higher concentration is observed in the standard strain of L. monocytogenes. This indicates that the higher the nanoparticle concentration, the greater its inhibitory effect.

5. Conclusion

In this study, the antibacterial effect of CNPs on L. monocytogenes, the causative agent of listeriosis, was investigated. The results showed that the nanoparticle has beneficial effects on the treatment of this disease in the body of a living organism (laboratory white mouse), and the higher the concentration of this nanoparticle, the greater its therapeutic effect. Because it has a natural origin, it is a suitable alternative to antibiotics, such as ampicillin, used to treat listeriosis, especially in pregnant women.

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References

Alebouyeh S, Assmar M and Mirpour (2020) Effect of Chitosan Nanoparticle from Penaeus semisulcatus Shrimp on Salmonella typhi and *Listeria monocytogenes*. Iran J Public Health, 49(2), 369–376.

Altuntas E.G, Kocan S, Cosansu S, Ayhan K and Juneja V.K (2012) Antibiotic and Bacteriocin Sensitivity of *Listeria monocytogenes* Strains Isolated from Different Foods. Food Nutr Sci, 3, 363-368.

Aranaz I, Mengíbar M, Harris R, Paños I, Miralles B , Acosta N , et al (2009) Functional characterization of chitin and chitosan. Curr Chem Biol, 3(2), 203-230.

Caruso M, Fraccalvieri R, Pasquali F, Santagada G, Latorre L, Difato L, et al (2020) Antimicrobial susceptibility and multilocus sequence typing of *Listeria monocytogenes* isolated over 11 years from food, humans, and the environment in Italy. Foodborne Pathog Dis, 17, 284–294.

Chavez LE , Resin A, Howard KA , Sutherland DS and Wejse PL (2011) Antimicrobial effect of chitosan nanoparticles on *Streptococcus mutans* biofilms. J Appl Environ Microbiol , 77, 3892-5.

Chen CS, Liau WY and Tsai GJ (1998) Antibacterial of N-sulfon-ated and N-sulfobenzoyl chitosan and application to oyster preservation. J Food Pro , 621(9),1124-1128.

Dutta P, Dutta J and Tripathi V.S (2004) Chitin and chitosan: Chimistry, Properties and applications. J Sci Ind Res , 63(1), 20-31.

Dutta P, Ravikumar MNV and Dutta J (2002) Chitin and chitosan for versatile applications. J Macromol Sci C - Polym Rev , 42(3), 307-354.

Feng Y and Xia W (2011) Preparation, characterization and antibac-terial activity of water-soluble O-fumaryl-chitosan. Carbo-hyd Polym , 83(3), 1169-1173.

Haginaka J, Wakai J, Yasuda H, Uno T, Takahashi K and Katagi T (1987) HighPerformance Liquid Chromatographic determination of Ampicillin and its metabolites in rat plasma, bile and urine by post-column degradation with sodium hypochlorite. J Chromatogr Open, 400, 101-111.

Haubert L, Mendonça M, Lopes G, de Itapema Cardoso M and da Silva W(2016) *Listeria monocytogenes* isolates from food and food environment harbouring tetM and ermB resistance genes. Lett. Appl. Microbiol, 62, 23–29.

Jeon Y, Park J and Kim K (2001) Antimicrobial effect of chito oligosaccharides produced by bioreactor. Carbohyd Polym , 44,71-76.

Jung W.C, Ha J.Y, Chung H.S , Heo S.H, Kim S and Lee H.J (2006) Application of a solid-phase fluorescence immunoassay to determine ampicillin residues in muscle tissue of olive flounder (Paralichthys olivaceus). Korean J Vet Res, 46, 291–294.

Jung S.H, Seo J.S and Park M.A (2012) Residues of ampicillin in blood of cultured olive flounder by oral, injection and dipping administration. Journal of fish pathology, 25(3), 211– 219.

Kazemi Rad S, Assmar M, Mirpour M and

Razavi M (2022) Evaluation of chitosan nanoparticle antimicrobial effect on isolated *Listeria monocytogenes* bacteria from pregnant women and *Listeria monocytogenes* ATCC 7644. Iran J Public Health , 51(12), 2783-2790.

Ketabchi M, Iessazadeh KH and 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. J of Food Microbiol,4(1), 63-74. Persian.

Ma Y, Liu P, Si C and Liu Z (2010) Chitosan nanoparticles: preparation and application in antibacterial paper. J Macromol Sci Phys ,49 (5), 994-1001.

Manso B, Melero B, Stessl B, Fernandez-Natal I, Jaime I, Hernández M, et al (2019) Characterization of virulence and persistence abilities of *Listeria monocytogenes* strains isolated from food processing premises. J Food Prot , 82(11), 1922-1930.

Mook P, O'Brien S and Gillespie I (2011) Concurrent conditions and human listeriosis, England, 1999-2009. Emerg Infect Dis, 17(1), 38–43.

Mota M, Vázquez S, Cornejo C, D'Alessandro B, Braga V , Caetano A, et al (2020) Does shiga toxin-producing *Escherichia coli* and *Listeria monocytogenes* contribute significantly to the burden of antimicrobial resistance in Uruguay .Front.Vet. Sci, 7, 903.

Pillai C, Paul W and Sharma C (2009) Chitin and chitosan polymers: chemistry, solubility and fiber formation. Prog Polym Sci, 34(7), 641-678.

Pizarro-Cerdá j and Cossart p (2019) Microbe Profile: *Listeria monocytogenes*: a paradigm among intracellular bacterial pathogens. Microbiology (Reading), 165(7), 719-721.

Radam S, Faleh I, Atshan O and Hasan M (2020) Effcacy of Chitosan Immune Response Against *Listeria Monocytogenes* Infection in Mice. Indian J Forensic Med Toxicol, 14(2), 2113-2216.

Steckel H and Nogly F(2004) Production of chitosan pellets by extrusion/spherinization. European J Pharm Biophar, 57(1),107-14.

Vazquez-Boland J, Kuhn M, Berche P, Chakraborty T, Domınguez-Bernal G, Goebel W, et al (2001) *Listeria* pathogenesis and molecular virulence determinants. Clin Microbiol Rev, 14(3),584-640.Wei D, Sun W, Qian W, Ye Y, Ma, X (2009) The synthesis of chitosan-based silver nanoparticles and their antibacterial ac-tivity. Carbohyd Res , 344(17), 2375-2382.

Yang K, Wang A, Fu M, Wang A, Chen K, Jia Q, et al (2020) Investigation of incidents and trends of antimicrobial resistance in foodborne pathogens in eight countries from historical sample data. Int. J. Environ. Res. Public Health , 17,472.

Zarei M, Basiri N, Jamnejad A and Eskandari M.H (2013) Prevalence of *Escherichia coli* O157:H7, *Listeria monocytogenes* and Salmonella spp. in beef, buffalo and lamb using multiplex PCR. Jundishapur J Microbiol, 6 (8), 1-5.

Zhang H , Wang J , Chang Z , Liu X , Chen , Yu Y , et al (2021) *Listeria monocytogenes* Contamination Characteristics in Two Ready-to-Eat Meat Plants From 2019 to 2020 in Shanghai. Front Microbiol, 12, 1-9.