



Research Journal of **Microbiology**

ISSN 1816-4935



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Establishment of Quantitative Real-Time PCR System for Analyzing Cas9 Gene Expression in *Listeria innocua*

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ABSTRACT

Currently, little is known about a protocol in establishing the quantitative Real-Time PCR (qRT-PCR) system in gram-positive bacteria. Also, it is obscure whether the expression level of clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (Cas9) in *Listeria innocua* is influenced by the pH. This study focused on providing a protocol for analyzing Cas9 gene expression in *L. innocua* at two pH conditions. The 37°C overnight culture was diluted 1:500 and grown in fresh Todd Hewitt Yeast extraction broth (THY) for 3 h. The collected pellet was washed two times in pH = 7.5 medium and was cultured for 4 h by using pH = 7.5 and pH = 6.0 medium, respectively. The culture was treated with RNA protection reagent for 5 min and the obtained pellet was suspended with mutanolysin for 30 min. Total RNA was extracted by using a kit and was incubated with DNase I, which was removed by adding phenol-chloroform-isoamyl alcohol thereafter. The purified RNA was precipitated via glycogen-ethanol treatment and was synthesized into cDNA. The $2^{-\Delta\Delta Ct}$ method was used to analyze the expression level of Cas9 gene with 16S rRNA gene as a reference. The gel electrophoresis showed that the quantity of 23S rRNA was about two-fold of 16S rRNA. The relative Cas9 expression levels of *L. innocua* in neutral and acidic medium were 1.08 ± 0.49 and 1.66 ± 0.54 , respectively ($p > 0.05$). Totally, the qRT-PCR system for analyzing Cas9 gene expression in *L. innocua* was successfully developed. The Cas9 expression level of *L. innocua* in acidic medium was similar to that in neutral medium.

Key words: *Listeria innocua*, gram-positive bacteria, Cas9 gene, quantitative real-time PCR

INTRODUCTION

The quantitative Real-Time PCR (qRT-PCR) technique has been applied widely in eukaryote species, especially in gene expression analysis of animal cells (Han *et al.*, 2006; Luo *et al.*, 2009). In the analysis of gene expression, fluorescent dye could bind different DNA templates and primers. Therefore, fluorescent dye based qRT-PCR is more popular because of its economy and practicality advantages. The technique is also used in identification of clinical pathogen, which usually executes DNA qualitative and quantitative identification via designing primers based on conserved sequence of bacteria gene (Xu *et al.*, 2007; Shu *et al.*, 2008). Currently, it has been reported about gene expression analysis of gram-positive bacteria based on the qRT-PCR technique. However, little is known about a step by step protocol in establishing the qRT-PCR system in gram-positive pathogens (Xu and Xu, 2013; Zou *et al.*, 2013).

The total RNA extraction is the initial step for analyzing gene expression via using qRT-PCR system. *Listeria innocua* belongs to the genus *Listeria* and is one kind of gram-positive bacteria. It is difficult to extract RNA from these kinds of bacteria due to the thick and tough cell wall. To extract RNA from these bacteria, high effective strategies in destroying the cell wall must be used. Moreover, there is no intron in bacteria genome, the RNA samples are easily contaminated by bacteria chromosomal DNA and the purification for RNA extraction is needed subsequently. Therefore, the cell wall destroying and RNA purification play crucial role in setting up a qRT-PCR system to analyze gene expression in bacteria.

The clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (Cas9) plays important role in bacterial immunity. Also, the published data indicated that Cas9 regulates the expression of virulence factor in transcriptional level in gram-negative bacteria (Sampson *et al.*, 2013). The environmental pH may influence the expression of virulence gene in gram-positive bacteria (Chiang-Ni *et al.*, 2012). Up to now, the function of Cas9 in different medium pH in gram-positive bacteria is still unknown. The aim of this study is to establish the qRT-PCR system in *L. innocua* and analyze the expression levels of Cas9 gene in neutral and acidic medium, respectively.

MATERIALS AND METHODS

Bacterial strain: *Listeria innocua* ATCC33090 strain was purchased from Beijing Zhongyuan Co., Ltd. (Beijing, China).

Major experimental reagents: Bacto™ Todd Hewitt Broth and Bacto™ Yeast Extract were purchased from Shanghai Huiyun Biological Technology Co., Ltd (Shanghai, China). HEPES and MES were purchased from Shanghai Xinfan Biological Technology Co., Ltd (Shanghai, China), respectively. RNaprotect® Bacteria Reagent and RNeasy® Mini Kit were bought from Shanghai Baili Biological Technology Co., Ltd (Shanghai, China). Mutanolysin and glycogen were purchased from Beijing Innochem Science and Technology Co., Ltd (Beijing, China). BioBRK RT Kit was bought from Chengdu BioBRK Co., Ltd (Chengdu, China).

Primer synthesis: 16S rRNA (F: 5'-GAGGGTCATTGGAAACTGGAA-3', R: 5'-CACTGGTGTTCCCTCCACATATC-3', band size = 97 bp) and Cas9 (F: 5'-CCGGAATGTTTGCTCA GTTTATC-3', R: 5'-CGCACATTCAATATCCGATTTCTC-3', band size = 98 bp) primers were synthesized by BioBRK Co., Ltd (Chengdu, China). iTaq™ Universal SYBR® Green Supermix was bought from BioRad (Chengdu, China).

Bacteria culture: Neutral medium and acidic medium preparation were performed as described previously (Chiang-Ni *et al.*, 2012). 100 mM HEPES and MES were used to adjust the fresh THY broth to pH = 7.5 (neutral medium) and pH = 6.0 (acidic medium) respectively. One single colony of *L. innocua* was inoculated into 4 mL neutral medium and was cultured overnight at 37°C. The bacterial culture was diluted 1:500 into 4 mL fresh neutral THY medium and was grown 37°C for 3 h. Then the pellet was collected by centrifugation at 4°C, 14000 rpm. The supernatant was discarded. Cells were suspended and washed by 1 mL neutral medium. After centrifugation, cells were suspended and cultured for 4 h by using neutral medium and acidic medium, respectively. After harvesting, cells were used to extract total RNA. The growth curves of *L. innocua* were performed as described previously (Shelburne III *et al.*, 2011). Growth was monitored by measuring A₆₀₀ at early exponential, mid-exponential, late exponential and stationary phases.

RNA preparation: Basically, the operation was followed the instruction of RNeasy[®] mini kit. It was slightly modified by adding cell-wall destroying, DNA contamination eliminating, precipitation and purification via using glycogen. All the pipette tips, tubes and deionized water used in this study were RNase-free. The main procedure was as follows:

One milliliter of RNAprotect[™] Bacteria Reagent was added directly to 500 μ L of bacterial culture and mixed immediately by vortex. Subsequently, the sample was incubated 5 min at room temperature and centrifuged 5 min at 14000 rpm. The supernatant was discarded (the pellet can be stored at -20°C for up to two weeks or at -70°C for up to four weeks for future use). For a subsequent operation, cells were suspended with 200 μ L RNase-free deionized water. Cell wall was digested by using 50 unit of mutanolysin for 30 min at 37°C, which was mixed every 2 min. Then 700 μ L of buffer RLT was added and mixed 2 min. After 1 min centrifuge at 14000 rpm, the supernatant was moved to a new tube and mixed with 500 μ L anhydrous alcohol (the sample can be stored at -20°C for up to two weeks). The purpose of these operations was to protect RNA from degrading, lyse cells and precipitate nucleic acid.

All of the above lysate was transferred into the RNeasy spin column and centrifuged 15 sec at 14000 rpm via repeating one time and the flow-through was discarded. By centrifuging at 14000 rpm for 15 sec, the sample was washed by adding 600 μ L buffer RW1 and 600 μ L buffer RPE to the RNeasy spin column subsequently. After discarding the flow-through, the RNeasy spin column was centrifuged for 2 min at 14000 rpm to remove residual ethanol. The RNeasy spin column was moved to a new 1.5 mL collection tube and the lid was opened to dry the membrane for 1 min. The operations of this stage were supposed to keep the nucleic acid in the column membrane.

The next steps in this stage were executed to purify total RNA. The 50 μ L RNase-free deionized water was add to the center of spin column membrane. After keeping 1 min at room temperature, total RNA was eluted via centrifuging for 1 min at 14000 rpm. To remove DNA contamination, the collected sample was mixed gently with four unit of DNase I and reaction buffer on ice and incubated at 37°C for 30 min subsequently. To remove protein contamination, the upper sample was mixed well for 15 sec with 450 μ L RNase-free water and 500 μ L phenol-chloroform-isoamyl alcohol (25: 24: 1) subsequently. After keeping 1 min at room temperature, the sample was centrifuged for 5 min at 14000 rpm. The supernatant was transferred to a new 1.5 mL centrifuge tube and mix well with 40 μ g glycogen and 1 mL ethanol subsequently. The mixed sample was stored at -20°C for 10 min or two weeks for future use. After discarding the supernatant, the sample was obtained via centrifuge for 10 min at 14000 rpm at room temperature. Carefully, the residual liquid was removed by using a pipette tip and the purified RNA was dried 2 min at room temperature. The RNA precipitation resolved and mixed gently with 20 μ L water. Immediately, the solution was used for a subsequent operation.

cDNA synthesis: The purity and concentration of total RNA were determined immediately by using ND-1000 (NanoDrop Technologies, USA). The quality of extracted total RNA was detected by running a 1.5% agarose gel electrophoresis. cDNA was synthesized quickly by using BioBRK RT Kit (BioBRK Co., Ltd. Chengdu, China). According to the manufacture's instruction, reverse transcription was performed on ice in a total volume of 20 μ L containing 500 ng RNA, 2.5 μ M of random hexamer primer, 1 \times RT buffer, 1 mM of each dNTP, 100 unit of ReverTra Ace, 5 unit of Super-RI, 0.5 μ L of RT Enhancer and RNase-free water to the final volume. The reaction mixture was incubated in 42°C for 40 min, followed by 5 min at 99°C to inactivate the enzyme. Synthesized cDNAs were diluted five times with sterile water and stored at -20°C until use.

Amplification of qRT-PCR: All samples were kept on ice and were avoided the lights. Cas9 gene of each cDNA sample was amplified in triplicate wells by using CFX96 Real-Time PCR Detection System (Bio-Rad, Richmond, CA). To reduce the pipette error, the common components were mixed together in one 1.5 mL tube and the total well number when preparing should be the exact well number plus one in reaction. The mixed solution was then distributed into each well of a 96 well plate (Bio-Rad, Catalog# HSP9631). At last, the diluted cDNA samples were added to each well. The plate was sealed with microseal B adhesive sealer® (Bio-Rad, Catalog# MSB1001) and the samples were mixed well via gently shaking. The sealed plate was centrifuged in 4°C for 2 min with 3750 rpm before executing the PCR amplification. The preparation of 16S rRNA gene amplification was similar to that of Cas9 gene. PCR reaction was performed in a total volume of 20 µL containing 10 µL of 2×Master Mix, 0.4 µL of each primer (10 µM), 4 µL diluted cDNA and 5.6 µL of RNase-free water. The thermal cycling profile used was consisted of 35 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C.

Data analysis: Comparison of transcript levels was performed using $2^{-\Delta\Delta C_t}$ method of analysis using 16S rRNA as the endogenous control gene. Triplicate biological replicates were grown on three separate occasions and analyzed in triplicate. All data were presented as Means±standard error of means (SDEM). The t-test was used to compare the statistic significance of relative quantity between neutral medium group and acidic medium group. A $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

With neutral medium and acidic medium treatment, the OD_{600} values of different culture were around 0.4. This indicated that the growth of *L. innocua* in medium with different pH was at early exponential phase. The similar growth curves were detected (Fig. 1). The data suggested that acidic medium have no effect on the growth of *L. innocua* in the present study. The concentration of all RNA samples isolated from *L. innocua* was $50 \text{ ng } \mu\text{L}^{-1}$ or so and the A_{260}/A_{280} value was 1.8-2.0. The gel electrophoresis showed that the quantity of 23S rRNA was approximately twofold compared with 16S rRNA (Fig. 2). These data indicated that the obtained total RNA was in a great quality. The transcript level of Cas9 in neutral medium and acidic medium was 1.08 ± 0.49 and 1.66 ± 0.54 , respectively ($p > 0.05$) (Fig. 3).

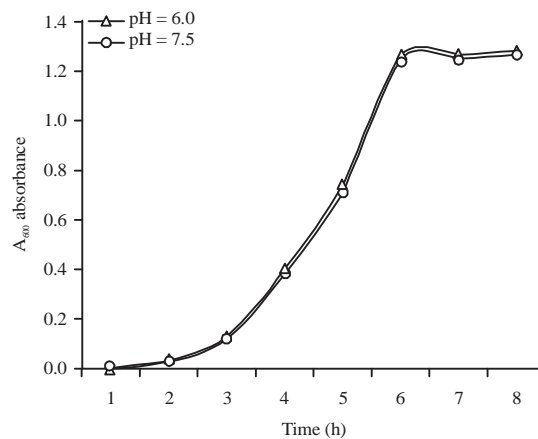


Fig. 1: Growth curve of *Listeria innocua* in pH = 7.5 and pH = 6.0 medium culture. Arrow indicates the sampling time point for Cas9 transcript analysis

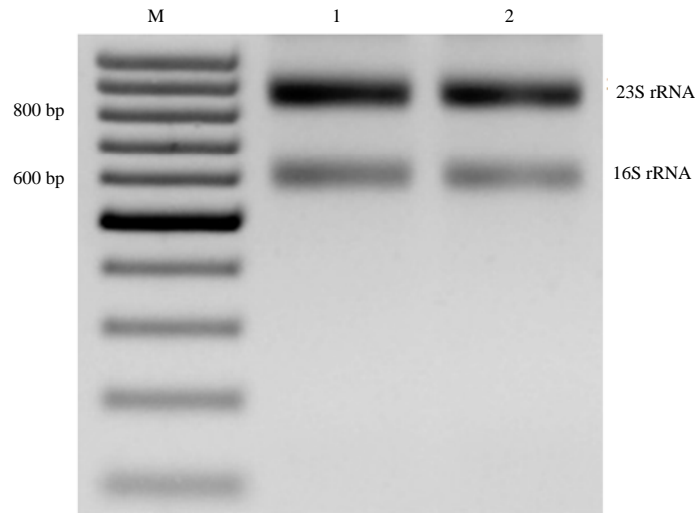


Fig. 2: Agarose gel electrophoresis of total RNA samples. 23S and 16S rRNA were indicated. Lane 1-2, total RNA sample extracted from *Listeria innocua* cultured in neutral medium and acidic medium, respectively. M, GeneRuler 100 bp DNA ladder

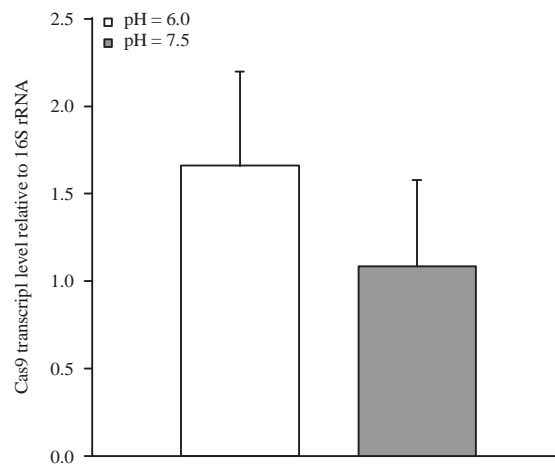


Fig. 3: Cas9 transcript level of *Listeria innocua* cultured in neutral medium and acidic medium ($p > 0.05$)

The extraction and purification of total RNA from bacteria are initial step for cDNA synthesis. As we all know, the cell wall of gram-positive bacteria is thick and has tight junction structure. Therefore, it is important to select an appropriate approach to extract gram-positive bacteria RNA by destroying the cell wall (Yang, 2006). Traditionally, total RNA is extracted from the lysate including different macromolecules in RNase denaturation chemical environment. However, the endogenous RNase still could be released and degrades the RNA in the process of lysis (Pang *et al.*, 2003). In the present study, total RNA is stabilized and its expression profile is fixed before the lysis via using RNA protection reagent. The gel electrophoresis data showed that total RNA was extracted with good purity and quality in this study.

Mutanolysin (N-acetyl muramidase) is a muralytic enzyme that cleaves the N- acetylmuramyl- $\beta(1-4)$ -nacetylglucosamine linkage of the bacterial cell wall polymer peptidoglycan-polysaccharide

(Liu *et al.*, 2000). Its carboxy terminal moieties are involved in the recognition and binding of unique cell wall polymers. Luo *et al.* (2008) found that the cell wall of *Bifidobacterium* is digested successfully by using mutanolysin. Similarly, the cell wall of *Listeria innocua* was also digested well to extract total RNA in the current study. Besides, the DNA contamination was eliminated by using DNase I. More RNA was precipitated via adding the glycogen, which promotes the quantity without disturbing the subsequent experiment (Sun *et al.*, 2005; Zheng *et al.*, 2007; Zhao *et al.*, 2009). Totally, the RNA extraction approach in this study could be executed in different stages and the time arrangement was flexible. Also, there was very little RNA degradation. This developed method could prepare perfect total RNA samples from gram-positive bacteria and thus could be used in subsequent gene expression analysis.

CRISPR/Cas (CRISPR-associated proteins) constitutes an adaptive RNA-mediated defense system which targets invading phages or plasmids (Deltcheva *et al.*, 2011). Cas9 belongs to type II CRISPR/Cas system and two endonuclease domains within Cas9 could execute cleavage of each strand of the targeted DNA (Sampson and Weisis, 2013). Recently, Sampson *et al.* (2013) demonstrated that Cas9 targets an endogenous transcript encoding an immunostimulatory bacterial lipoprotein (BLP), leading to mRNA degradation and decreased transcript levels. The low BLP level is helpful in avoiding the activation of a Toll-like Receptor 2 (TLR2)-dependent proinflammatory response in host, which facilitates the infection of *Francisella novicida*, a gram-negative bacterium. Therefore, Cas9 also plays a crucial role in infection process of bacteria via regulating virulence factor. Chiang-Ni *et al.* (2012) indicated that environmental pH changes regulate the expression of virulence factor SpeB in *Streptococcus pyogenes*. However, little is known about whether the expression of Cas9 is influenced by medium pH in gram-positive bacteria. In the present study, no significant difference was detected in the relative Cas9 expression levels of *L. innocua* in neutral and acidic medium. This suggested that the main function of Cas9 is for bacterial defenses against foreign genetic elements derived from bacteriophages, plasmids, or extracellular chromosomal DNA, which is similar in different environmental pH situation.

The quantitative Real-Time PCR system for analyzing Cas9 gene expression in *L. innocua* was established successfully in the current study. This approach provides the protocol for analyzing gene expression in gram-positive bacteria. The obtained data also displayed the reference for the expression level of Cas9 in acidic medium.

ACKNOWLEDGMENTS

The authors contribute equally to the present work. We are thankful to the Key Project of the Education Department of Sichuan Province (No. 12ZA241), the Project of the Health Department of Sichuan Province (No. 120379) and the joint Scientific Research Project of Science and Technology Department of Sichuan Province, Luzhou People's Government and Sichuan Medical University (No. 14JC0089).

REFERENCES

- Chiang-Ni, C., P.X. Zheng, P.J. Tsai, W.J. Chuang, Y.S. Lin, C.C. Liu and J.J. Wu, 2012. Environmental pH changes, but not the LuxS signalling pathway, regulate SpeB expression in M1 group A streptococci. *J. Med. Microbiol.*, 61: 16-22.
- Deltcheva, E., K. Chylinski, C.M. Sharma, K. Gonzales and Y. Chao *et al.*, 2011. CRISPR RNA maturation by *trans*-encoded small RNA and host factor RNase III. *Nature*, 471: 602-607.
- Han, C., D. Zhao, C. Wu, Z. Ning and J. Yang *et al.*, 2006. Quantification of sheep prion gene expression using real-time RT-PCR. *J. China Agric. Univ.*, 11: 61-64.

- Liu, T., W. Xu and Y. Zhang, 2000. History and prospects of the research on mutanolysin. *Acta Microbiol. Sin.*, 40: 224-227.
- Luo, Y.L., X.P. Huang, X.M. Wu, S.T. Ding and Y.P. Ma *et al.*, 2008. Studies on extraction of bifidobacterium plasmid by mutanolysin from *Streptomyces globisporus* fermentation fluid. *Chin. J. Microecol.*, 20: 527-528.
- Luo, C., Z.Q. Wang, F.Q. Gong and D.S. Shi, 2009. Detection of histone acetylation related gene expression in buffalo somatic cells by SYBR Green real-time fluorescence quantitative PCR. *Chin. J. Vet. Sci.*, 29: 233-237.
- Pang, X., D. Zhou and R. Yang, 2003. Summary of bacteria mRNA extraction. *Biotech. Bull.*, 1: 30-34.
- Sampson, T.R. and D.S. Weiss, 2013. Alternative roles for CRISPR/Cas systems in bacterial pathogenesis. *PLoS Pathog*, Vol. 9. 10.1371/journal.ppat.1003621
- Sampson, T.R., S.D. Saroj, A.C. Llewellyn, Y.L. Tzeng and D.S. Weiss, 2013. A CRISPR/Cas system mediates bacterial innate immune evasion and virulence. *Nature*, 497: 254-257.
- Shelburne III, S.A., R.J. Olsen, N. Makthal, N.G. Brown and P. Sahasrabhojane *et al.*, 2011. An amino-terminal signal peptide of Vfr protein negatively influences RopB-dependent SpeB expression and attenuates virulence in *Streptococcus pyogenes*. *Mol. Microbiol.*, 82: 1481-1495.
- Shu, X.L., Y.D. Wu and S.Q. Shang, 2008. Establishment of quantifying and typing analysis of 16S rRNA gene by real-time PCR. *Chin. J. Contemp. Pediatr.*, 10: 732-736.
- Sun, Y.X., H.M. Sun, R.L. Guo, X.H. Zhang and J.Q. Yuan, 2005. Isolation of total RNA from human lens capsules and cultured lens epithelial cells with modified one-step method *Chin. Ophthalmic Res.*, 23: 293-296.
- Xu, D.S., X.F. Wu and P.Q. Cheng, 2007. Comparison of real-time fluorescence PCR with PCR and bacterium culture in detection of *Listeria monocytogenes*. *Chin. J. Health Lab. Tech.*, 17: 861-863.
- Xu, S. and X. Xu, 2013. An improved method suitable for extraction of total RNA from *Bacillus subtilis*. *J. Northeast Agric. Univ.*, 44: 36-41.
- Yang, R.D., 2006. Foundations of Modern industrial Microbiology. Higher Education Press, Beijing, China, Pages: 435.
- Zhao, J., Z.C. Liu, L. Dai and M.J. Liu, 2009. Isolation of total RNA for different organs and tissues of *Ziziphus jujuba* mill. *J. Plant Genet. Resour.*, 10: 111-117.
- Zheng, K., Q.S. Chen and X. Li, 2007. A method established for total RNA isolation from the body wall of *Apostichopus japonicus*. *Lett. Biotech.*, 18: 84-87.
- Zou, X.L., L.C. Liu and L.X. Luo, 2013. Comparison of different methods for total bacteria RNA extraction. *Modern Food Sci. Tech.*, 29: 1948-1954.