MODIFIED TECHNIQUES IN QUANTIFICATION OF INTRACELLULAR LISTERIA MONOCYTOGENES IN VITRO INFECTION

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Received: 03.8.2019
Accepted: 27.12.2019

SUMMARY

The demand for reliable methods for the quantification of intracellular bacteria is growing. Among modern methods such as PCR and flow cytometry, traditional methods including colony forming unit assay and immune-fluorescence are still the two most commonly techniques worldwide. In colony forming unit assay, there are variations among publications, making data results inconsistent across studies. The aim of this paper is to evaluate available techniques and develop improved protocols for the quantification of intracellular Listeria monocytogenes (LM) in vitro infection assay. This study has suggested different uptake time for phagocytic and non-phagocytic cells. Specifically, uptake time was determined at 0.5 hour after infection for RAW264.7 macrophages and 2 hours for L929 fibroblast host cells. To efficiently remove extracellular bacteria during infection period, gentamicin at high and low concentrations was used during the infection assay. High concentration of gentamicin was used to kill extracellular bacteria while low concentration of gentamicin was used to prevent secondary infection of host cells during the infection period. To obtain a more accurate number of alive LM from a large scale experiment, phosphate-buffered saline/PBS should be used rather than multi-Q (mQ) water to lyse the host cell as mQ water can kill additional bacteria unexpectedly. In immune-fluorescence, LM can be visualized by using either the LM expressing green fluorescence protein (GFP) or antibody against LM. To observed GFP signal, cells should be fixed with paraformaldehyde as methanol will rapidly dim the GFP signal. Findings from this study will benefit researchers engaged in both basic cell biology and infectious diseases.

Keywords: bacteria, colony forming unit, intracellular, Listeria monocytogenes, macrophages

INTRODUCTION

Listeria monocytogenes (LM) is a gram positive food-borne intracellular bacterium. Healthy people once infected by LM usually got mild to severe gastroenteritis. However, in immune-compromised and susceptible individuals including the elderly, the new-born and pregnant women, very low numbers of ingested bacteria (from $10^2$ to $10^4$) can cause listeriosis. The life-threatening listeriosis can lead to systemic infection followed by meningitis. In addition, listeriosis in pregnant women might cause abortion or fetal complications. Despite rarely occurrence, listeriosis is responsible for up to 20 - 30% of mortality in vulnerable patients (de Noordhout et al., 2014). Particularly, LM can survive under food storage conditions, such as cold temperature (4°C), high pH and high salted levels (Cossart, 2011), which makes this bacterium as an alarming food pathogen (Ferreira et al., 2016).

LM can infect various cell types from non-phagocytic cells (epithelial cells, hepatocytes, endothelial cells, fibroblasts) to phagocytic cells (monocytes, macrophages, neutrophils) (Hamon et al., 2006). Inside the host cells, LM express multiple virulence factors that allow them to escape host vacuoles, replicate intracellularly and spread to neighbouring cells (Vázquez-Boland et al. 2001).

As LM is an easily-grown bacterium and its pathogenicity has provided a plenty of useful knowledge of host-pathogen interaction, studying pathogenesis of LM during its intracellular invasion of host cells has become a special interest to immunologists (Becattini et al., 2017).
Quantification of intracellular LM is an important step, which allowing an overview of host cell capability to deal with this professional bacterium during phagocytosis. The advent of molecular tools allows the enumeration of intracellular LM to be performed by several approaches, including colony-forming unit (CFU) on agar plates (Portnoy et al., 1988), immunofluorescent staining of LM (Drevets, Campbell, 1991), PCR methods (qPCR and dPCR) (Traunick et al., 2011; Ricchi et al., 2017) and flow cytometric quantification of intracellular bacteria (Swarts et al., 1998). Of those, the first two methods are mostly used in published studies, probably due to their cost-effectiveness and visual data presentation. Although CFU performance is a standard and popular technique, for intracellular quantification of LM, different papers have displayed a slightly different step during CFU procedure, which may affect the accuracy of data published. This paper will analyze the method of CFU quantification of intracellular LM based on published studies, as well as provide some modified steps during CFU procedure to acquire more precise data. In accordance with providing an improved CFU protocol, some useful notes during immune-fluorescence were also provided.

MATERIALS AND METHODS

Bacteria preparation

Three to four single bacterial colonies of LM strains (wild type 10403S, GFP-expressing LM (10403S) (Shen, Higgins, 2005) were added to 5 mL of Brain Heart Infusion/BHI broth, incubated at 37°C overnight in a rocking shaker at 250 rpm, 37°C to obtain an OD600nm between 1.4 to 1.6. The bacterial culture was then diluted 1/100 in 10 mL of BHI broth, and further shaken in the rocking shaker for 2 hours (h) to obtain an OD600nm from 0.05 to 0.1 (Myers, Tsang et al., 2003). Bacteria were centrifuged at 3273 x g at 4°C for 10 minutes (min). The pellet was resuspended and diluted in pre-warmed cell culture medium to obtain the desired multiplicity of infection (MOI) before adding to cells. For MOI check, LM inoculum was plated as 10-fold serial dilutions (10⁻³, 10⁻⁴, 10⁻⁵) on BHI agar plates. These plates were incubated at 37°C from 24 to 48h, and colonies counted to determine colony forming units (CFU).

Listeria monocytogenes infection of macrophages

LM infection of the macrophage cell line RAW264.7 was performed as described previously (Tilney, Portnoy, 1989). Cells were seeded into 48-well plates at 1.5 × 10⁵ per well overnight in complete medium, which contains Dulbecco's Modified Eagle Medium/DMEM supplemented with 10% FBS, 2 mM L-Glutamine, 1 mM Sodium Pyruvate and 10 mM HEPES buffer in DMEM (all GIBCO). Cells were infected with LM at MOI 5 and centrifuged at 335 x g for 2 min at RT. Infected cells were incubated at 37°C and 5% CO₂. At 0.5 h post infection (p.i.), cells were washed twice with 50 µg/mL gentamicin diluted in DMEM to kill extracellular LM (Kuhn et al., 1988). Cells were washed twice with warm PBS and further incubated with complete DMEM supplemented with 5 µg/mL gentamicin to prevent continual re-infection of macrophages by LM released from dying cells. At different periods after infection, cells were washed once with 0.5 mL warm PBS and lysed in 1 mL of sterile 0.1% Triton X-100 in PBS. Numbers of viable intracellular LM were determined by performing 10-fold serial dilutions and plating on BHI agar plates. Aliquots of 20 µL of undiluted, 10⁻¹, 10⁻², 10⁻³ diluted lysate in PBS were spread on BHI agar plates (1.5% agar). Plates were incubated at 37°C from 24 to 48h and CFU was counted.

Immunofluorescence

Sterile glass coverslips 15 mm ø (G420-15, ProSciTech) were put into each well of a 24 well plate. Macrophages were seeded at 2 x 10⁵ cells per well in 350 µL complete medium one day prior to infection. Macrophages were infected with LM at MOI 3 (1 macrophage: 3 LM). At 0.5h after infection, cells were washed twice with 50 µg/mL gentamicin diluted in DMEM to kill extracellular LM. Cells were washed twice with warm PBS and further incubated with complete DMEM supplemented with 5 µg/mL gentamicin. At different time points after infection, cells were fixed by 4% paraformaldehyde diluted in PBS for 15 min at room temperature. Fixed cells then were washed twice with PBS and blocked for 1h in blocking buffer (2% bovine serum albumin/BSA containing 0.1% Triton X-100/PBS) at room temperature. After blocking, cells were washed three times with PBS and stained for 1h with the primary antibody against CD11b-PE (diluted at 1:200) (Biosciences) to visualize macrophage surface membrane. Cells then were washed with PBS and stained with the secondary antibody Alexa flour 594-conjugated donkey-anti-rabbit IgG (5 µg/mL, Abcam) for 1h in the dark. Primary and secondary antibodies

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were diluted blocking buffer. To visualize polymerized actin, after fixation step, cells were stained with Alexa fluor 467-conjugated Phalloidin (6.6 x 10^{-3} µM) (Cell Signaling Technology). Cell nuclei was stained with 4’, 6-diamidino-2-phenylindole dihydrochloride/DAPI (1 µg/mL, Molecular Probes). After staining, the coverslips were washed three times with PBS and one time with distilled water to remove residual salts. Coverslips were mounted with ProLong Gold Antifade Mountant (Life Technologies) and coverslip edges were sealed on microscope slides (S21102A Menzel) using clear nail polish. Microscopy slides were examined using an Olympus Epifluorescence inverted microscope IX73 (60 x magnification). Scale bars represent 10 µm.

RESULTS AND DISCUSSION

**Determination of time point when macrophages take up LM**

Determination of a right time point when host cells just about complete the internalization of LM is important, as earlier than that point the bacteria still locate extracellularly but later than that point the intracellular bacteria are replicated or partially killed by the host. An uptake point was determined by the first washing time stated in the study. In fact, differential uptake time points were set up by different studies, which largely depending on whether host cells are phagocytic or non-phagocytic cells (e.g. Caco-2, Hela, MEF). In studies with host cells are macrophages, which rapidly engulf LM via phagocytosis pathway (Radoshevich, Cossart, 2017), uptake time is usually set up from 30 min (Portnoy et al., 1988; Kuhn et al., 1988; Birmingham et al., 2007; Woodward et al., 2010) to 45 min (de Chastellier et al., 1994) after infection. In this study, uptake time point was observed since at 0.5h post infection of macrophages (Figure 1A). At 0.5h post infection, most of macrophages engulfed from 1 - 2 bacteria per cell whereas at 2h post infection, there are more bacteria per cell and most of cells contain more than 15 bacteria (Figure 1B). As the infection was halted at 0.5h post infection by gentamicin wash, the increase number of bacteria at 2h compared to 0.5h indicates that at 2h post infection, intracellular bacteria already multiply.

When host cells are non-phagocytic cells such as Caco-2, Hela, MEF, which internalize LM through receptor-mediated endocytosis (Cossart, Helenius, 2014), it requires at least 60 min for the cells to take up LM (Gaillard et al., 1987; Portnoy et al., 1988; Py et al., 2007). However, in our LM infection assay of L929 cells, at 60 min after infection, majority of bacteria still bind to the cell membrane. Extension uptake time until 2h post infection, intracellular LM was observed (Figure 2). Therefore, the uptake time point might be extended depending on the host cell types. To optimize the uptake, after adding bacteria onto host cells, the plate can also be centrifuged, as modified by Birmingham et al. (2007). In this study, after adding LM onto the cultured host cells, including macrophages and other non-phagocytic cells, the plate containing those infected cells were centrifuged at 335 x g for 2 min at room temperature to synchronize the uptake.

**Using gentamicin at different concentrations for different purposes**

Gentamicin, which cannot go through mammalian cells, was largely used to kill extracellular or adherent LM not removed by the first washing step (Devenish et al., 1981). There are different concentrations of gentamicin used in LM infection assays. Whereas in most of studies, gentamicin at 5, 10 or 50 µg/mL were added to the cell media during the experiment duration after washing (Pornoy et al., 1988; Kuhn et al., 1988; Woodward et al., 2010; Birmingham et al., 2007), other studies used gentamicin at 5 or 10 µg/mL to wash the extracellular bacteria in the first washing step (Py et al., 2007; Gaillard et al., 1987). It is demonstrated that to wash off adherent bacteria and prevent re-infection by bacteria released after cell burst, two concentrations of gentamicin can be used in the infection assay. Firstly, at 30 min post infection for host cells are macrophages or 60 min or so for other non-phagocytic cells, cells were washed twice with 50 µg/mL gentamicin diluted in DMEM to kill extracellular LM. Cells were washed twice with warm PBS and further incubated with media supplemented with 5 µg/mL gentamicin to prevent continual re-infection of host cells by LM released from dying cells.

**Triton X-100 diluted in PBS is used to release intracellular LM whereas distilled water (mQ water) unexpectedly killed further bacteria**

At different periods after infection, cells were washed once with warm PBS and lysed in 0.1% Triton X-100 in PBS. To lyse the cell for the releasing of intracellular LM, several studies use sterile mQ water (Gaillard et al., 1987; Pornoy et al., 1988; Py et al., 2007; Woodward et al., 2010) whereas others
used Triton X-100 (Kuhn et al., 1988; de Chastellier et al., 1994; Birmingham et al., 2007). For a more rapid lysing of host cells, ice-cold 0.1% Triton X-100 was efficiently used to lyse the cells after the final wash. It is noticeable that the number of viable intracellular LM dropped significantly when lysing host cells in Triton X-100 diluted in mQ water for 1.5h before plating. However, Triton X-100 diluted in PBS did not kill more LM at 1.5h compared to 0.5h. This suggested that LM cannot resist to water for long time incubation. Therefore, it will give a more reliable CFU data when using 0.1% Triton X-100 diluted in PBS to lyse the cell than using mQ water, especially in large-scale experiments which require more extensive time for washing, lysing and plating the lysate.

Figure 1. A. RAW264.7 mouse macrophages take up LM since at 0.5h post infection. B. Display of bacteria numbers inside infected cells at 0.5h and 2h post infection. (A-B: Data are means ± SEM from one experiment of the three, performed in triplicates, analyzed in Graphpad Prism).

Figure 2. *Listeria monocytogenes* infection of L929 cells, uptake was determined at 2h post infection. Intracellular bacteria were captured at 4h post infection.
Figure 3. Decrease of viable LM in lysate when lysing the cells with mQ water. RAW264.7 macrophages were infected by LM at MOI 5. Data are means ± SEM from one experiment of the three, performed in triplicates, analyzed in Graphpad Prism.

Figure 4. A. Methanol degraded GFP signal. B. LM can be stained using antibody against LM. A-B. RAW264.7 macrophages were infected with GFP-LM (A) or wild type LM (B) at MOI 3. LM was stained using primary antibody goat anti-Listeria (KPL, diluted at 1:100) and secondary Donkey Anti-Goat IgG H&L (Alexa Fluor® 594) (Abcam, diluted at 1:400).
Using immunofluorescence technique to quantify intracellular bacteria

Immunofluorescence imaging of bacteria is also a standard procedure allowing the quantification of intracellular bacteria. Preliminary studies utilized ethidium bromide to stain extracellular LM in red, which distinguished from the green-fluorescent intracellular LM in infected macrophages (Drevets, Campbell, 1991). This technique is fairly simple but it requires more time working with the microscope to switch from one light source or filter to another. With the modified protocol of gentamicin treatment, most of extracellular bacteria were washed off, leaving intracellular bacteria alive. RAW264.7 macrophages were infected with LM expressing GFP. After 0.5h, cells were washed with gentamicin (50 μg/mL in PBS) to remove extracellular bacteria and fresh medium containing gentamicin (5 μg/mL) was replaced and cultures maintained until the time of interested. Cells were fixed with paraformaldehyde 4% and stained with DAPI to observe nuclear DNA and with other markers to visualize the host cell membrane or boundary. In this study, CD11b, which expressed on the macrophage surface, was used to stain the cell (Figure 1A). In addition, Phalloidin can also be used to stain polymerized actin, which allowing the visualization of the host cell cytoskeleton (Figure 2). It is important to notice that to observe GFP-LM, cells should be fixed with paraformaldehyde, not with absolute methanol. Methanol is also a fixable reagent which is commonly used in immunofluorescence microscopy; however, methanol promptly ablates the fluorescence signal of GFP protein thus intracellular GFP-LM cannot be observed with methanol fixation (Figure 4A). To stain other proteins which require methanol fixation, it is advisable to use antibody to stain LM instead of using GFP-LM for the infection (Figure 4B).

CONCLUSION

This study gives an overview of using CFU and immunofluorescence as standard techniques to quantify intracellular LM. Published papers have been using different protocols for in vitro infection of LM and CFU performance, making it difficult to interpret the data. During macrophage infection of LM, uptake time was determined at 0.5h post infection. Different concentrations of gentamicin were used during the infection assay to kill extracellular bacteria and prevent continual infection effectively. To lyse the host cells and release intracellular bacteria for colony counting, PBS should be used in charge of mQ water in large scale experiments. In immunofluorescence, LM can be visualized by using either the LM expressing GFP, or an antibody against LM. To observe intracellular GFP-LM, cells should be fixed with paraformaldehyde as methanol will rapidly quench the GFP protein.

Acknowledgment: This project is supported by Australia Awards Scholarships. I sincerely thank Associate Professor Antje Blumenthal and all members of Blumenthal lab, Diamantina Institute, School of Medicine, the University of Queensland for supporting me during my PhD.

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muốn. Trong thí nghiệm nhuộm huỳnh quang, LM có thể được quan sát bằng cách sử dụng dòng vi khuẩn phát quang hoặc dùng kháng thể huỳnh quang để nhuộm vi khuẩn. Khi sử dụng dòng vi khuẩn phát quang, tế bào chỉ cần được cất bằng paraformaldehyde thay vì dùng methanol vì methanol sẽ làm hư hại các protein phát quang. Các kết quả của bài báo này mang lại nhiều thông tin tham khảo thiết thực trong nghiên cứu về tế bào học và bệnh truyền nhiễm.

Từ khóa: đại thực bào, đơn vi khuẩn lạc, Listeria monocytogenes, nội bào, vi khuẩn