BA-ELISA isolation of cytosolic *Salmonella* and vacuolar *Salmonella*

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Abstract：*Salmonella* is a pathogenic Gram-negative bacterium and important zoonotic pathogen survive in host cell, which is well known to cause gastrointestinal infections and even death in human through food contamination. *Salmonella* survives and reproduces within cells in two forms of cytosolic *Salmonella* and vacuolar *Salmonella*. However, the current researches on the mechanism of *Salmonella* infection was mostly based on the total numbers, and there is no clear distinction between cytosolic *Salmonella* and vacuolar *Salmonella*. This article combined biotin-streptavidin-amplified enzyme-linked immunosorbent assay(BA-ELISA) and Flat colony counting method to separate and quantify cytosolic *Salmonella* and vacuolar *Salmonella.* The results showed that the anti-*Salmonella* antibody with a dilution ratio of 1:500 can bind ~1700 *Salmonella*, and the binding rate reached to~70%. In terms of bacterial counts, vacuolar *Salmonella* dominates in the early stage of *Salmonella* infection, and cytosolic *Salmonella* dominates in the later stage, the reproduction rate of vacuolar *Salmonella* is significantly lower than cytosolic *Salmonella* during *Salmonella* infection, the dynamic growth curve of cytosolic *Salmonella* and vacuolar *Salmonella* intuitively reflected the proliferation status of the two forms of *Salmonella* during the entire process of *Salmonella* infection.

1 Introduction

Salmonella, belonging to Gram-negative facultative anaerobes, is one of the important pathogenic bacteria causing food poisoning and the most studied human pathogen[1]. The pathogenicity of *Salmonella* depends on its ability to survive in nonphagocytic epithelial cells[2]. There are two ways of Salmonella surviving in the epithelium. One is surviving within the *Salmonella* containing vacuole (SCV)[3] and the other is for escaping from within the membrane compromised SCV to acquire abundant nutrients in the cytoplasm and propagating at a faster rate for *Salmonella*[4]. At present, much of the researches about the propagation mechanisms of intracellular *Salmonella* are still at the level of total bacterial counts, and the distinction between *Salmonella* within the SCV and free *Salmonella* within the host cytoplasm has been in an ambiguous area. Experimenters have used fluorescently labeled *Salmonella* to infect macrophages for fluorescent screening and enrichment of intracellular pathogens by flow cytometry, but the shearing forces caused by flow cytometric sorting can lead to serious damage to the pathogens[5]. by paramagnetic nanoparticle labelled *Salmonella* to infect macrophages, Vikash et al. successfully isolated intact SCVs under the action of a magnetic field in 2018[6]. However, unlike macrophages, *Salmonella* produce *Salmonella* inducible filaments (SIFs) late in maturation within the epithelium assisting in SCV localization to the perinuclear region near the Golgi[7-8], further limiting the range of activity of *Salmonella* within the SCV and preventing isolation of SCVs within the epithelium by the same method. Thus, hyper-replication *Salmonella* within the cytoplasm emerge as another breakthrough in isolating and quantifying the two types of *Salmonella* within epithelial cells.

Biotin streptavidin amplified enzyme linked immunosorbent assay (BA-ELISA) is an established detection technique based on the principle of conventional ELISA combining the highly amplified effect between biotin and avidin with high sensitivity[9-10]. A horseradish peroxidase (HRP)-conjugated avidin molecule reacts with an anti-*Salmonella* conjugated biotin molecule to act as a multistage amplification[11-12], and HRP reacts with 3,3′, 5,5′ - tetramethylbenzidine (TMB) to produce a color change[13], ultimately achieving the goal of quantifying *Salmonella*. In this study, the concentration of anti-*Salmonella,* anti-*Salmonella* (biotin), antigen antibody binding time will be optimized to further quantify the amount of *Salmonella* binding to the antibody. The effective separation and quantification of intracellular free *Salmonella* from SCV coated *Salmonella* based on BA-ELISA, and the improvement of the separation method of host intracellular SCV from free *Salmonella* will provide a theoretical basis for further related studies to explore the pathogenic mechanism of *Salmonella*.

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2 Materials and methods

2.1 Bacterial strains

Salmonella enterica serovar Typhimurium LT2 is preserved in the General Microbiology Center of China microbial strain Preservation Management Committee (CGMCC), No.7020.

2.2 Optimization of the BA-ELISA procedure

The bottom of the wells of an ELISA plate was coated with a polyclonal rabbit anti-*Salmonella* antibody(1:50, 1:100, 1:250, 1:500, 1:1000, 1:2000, 1:400, 1:8000) and Anti-*Salmonella* (biotin) antibody(1:50, 1:100, 1:250, 1:500, 1:1000, 1:2000, 1:400, 1:8000) in PBS and incubated overnight at 4°C. The optimal concentration of antibody was optimized by using the numbers of *Salmonella* determined by flat colony counting method. After that, the antibody was co-incubated with quantitative *Salmonella* at 4°C for 0.5h, 1h, 2h and 4h, and the numbers of conjugated *Salmonella* were used as the intuitive judgment index for optimization.

2.3 Procedures of BA-ELISA experiment

The microtiter plate wells were coated with 100μL of the coating antigen(anti-*Salmonella* antibody) in coating buffer followed by overnight incubation at 4°C. After four washings with PBST (PBS with 0.05% Tween-20), Blocking was carried out by incubating the 200μL/wells with a 2% BSA solution at room temperature for 90min. The solution was discarded, and the plates were washed, as described above. Then, 100μL of the standard solution were added at 4°C for 2h, after another washing step, 100 μL/well anti-Salmonella (biotin) antibodies were added and incubated at 37°C for 1h. The plates were washed again, 100 μL/well of streptavidin-labeled HRP, at a dilution of 1:1000 dilution buffer, was added, and the plates were incubated at 37°C for 30 min. The TMB was added at 100μL/well after a final washing step, and the enzymatic reaction was incubated for 10min at 37°C and then was terminated by the stop solution (100 μL/well). The absorbance of each well was measured at 450nm.

2.4 Cell culture and infection

Henle-407 cells were cultured in antibiotic-free Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine fetal serum (FBS) at 37°C under a 5% CO_2 atmosphere. The cells were digested and passaged with 0.25% trypsin. Cells at a confluence of 80% were starved in FBS-deficient DMEM for 2 h. Starved cells were then infected with *S.* Typhimurium at an MOI of 20 for 30min. After washingthree times with PBS, the infected cells were subsequently incubated with DMEM and 100μg/mL gentamicin for 30 min. Finally, the infected cells were washed once with prewarmed phosphate-buffered saline (PBS) and then incubated with DMEM and 10μg/mL gentamicin until the harvest times.

2.5 Counting of total intracellular *Salmonella*

When the cells were infected with *Salmonella* for 2h, 4h, 8h and 16h, the cells were washed with PBS for three times and digested by trypsin. The cell precipitates were obtained by 800g centrifugation 5min at 4°C. The collected cells were washed three times by ice bath cleavage buffer (250mM sucrose, 0.5mM EGTA, 20mM HEPES-KOH (pH 7.4), 150mM NaCl, 1mM PMSF), all steps were performed at 4°C. Between 30 and 40 strokes were performed, until more than 80% free nuclei were visible. Nuclei and intact cells were removed by performing three sequential centrifugations, at 100g(1000rpm) for 5min each, in order to obtain the PNS. The PNS was divided into two parts, one was used for BA-ELISA to separate vacuolar *Salmonella* and cytosolic *Salmonella*, the other was extracted with a low osmotic solution (250mM sucrose, 0.5mM EGTA, 20mM HEPES-KOH (pH 7.4), 150mM NaCl, 1mM PMSF, 0.2% Triton X-100) for secondary extraction, and the total numbers of *Salmonella* was obtained by flat colony counting method.

2.6 ELISA for quantification of cytosolic Salmonella and vacuolar *Salmonella*

Samples from the PNS were added to the wells and incubated for 2h at 4°C, the experimental procedures were same as 2.3. The vacuolar *Salmonella* bound in the well by BA-ELISA, and the numbers of total *Salmonella*, vacuolar *Salmonella* and cytosolic *Salmonella* were calculated respectively.

2.7 Statistical analyses

Statistical significance was calculated by a one-tailed distributed paired Student t test or by two-way analysis of variance (ANOVA) with Origin 8.6 and SPSS 11.5， results are presented as the means ±SD for three independent experiments. p values of < 0.05 were considered to indicate significant differences.

3 Results

3.1 Optimization of the BA-ELISA procedure

After radiation treatment of highly adsorptive 96-well plate, polystyrene binds to antibody stably through hydrophobic interaction and ion interaction, and the binding of antigen and antibody enables *Salmonella* antibody to bind to quantitative *Salmonella*. The numbers of *Salmonella* bound to antibodies was indirectly obtained by flat colony counting (Fig.1). With the decrease of the dilution ratio of anti- *Salmonella* antibody and anti-*Salmonella* (biotin) antibody, the numbers of *Salmonella* bound by antibody increased significantly, which was proportional to the concentration of antibody. With the increase of antibody concentration, the binding amount of *Salmonella* -antibody reached a certain saturation, the numbers of *Salmonella* bound by anti-*Salmonella* antibody was about 3200 CFU, while the numbers of

Salmonella bound by anti-*Salmonella* (biotin) antibody was about 2700 CFU. Based on the principle of saving antibodies, and in order to ensure the accuracy and feasibility of the experiment, comprehensively considering the needs of the experiment, the concentration of antibody under the dilution of 1:500 as the best concentration for the experiment.

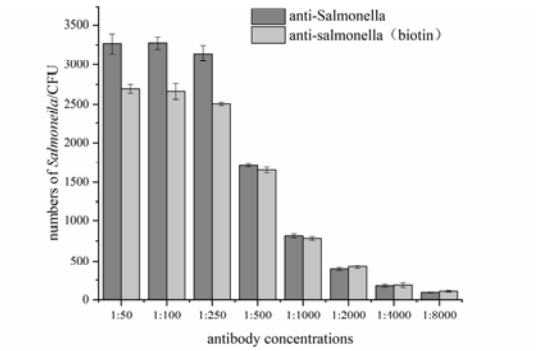

Figure 1. The optimization of anti-*Salmonella* and anti-*Salmonella* (biotin) antibody concentrations

By comparing the binding amount and binding rate of *Salmonella* obtained from different incubation time (Table 1), we found that the binding amount of *Salmonella* increased with the extension of incubation time, but there was no significant difference in the ratio of *Salmonella* that the antibody could bind between 2h and 4h($p<0.05$). Therefore, the best incubation time for *Salmonella* to bind with antibody at 4℃ is 2h.

Table1. effect of incubation time on the ability of antigen-antibody binding

Incubation time/h	Total bacteria count	Binding capacity	Binding rate
0.5	459±46.34	134±24.74	29.12%± 0.62%[a]
1	714±68.94	269±50.72	37.68%± 1.18%[b]
2	1232±38.08	862±79.15	69.96%± 0.78%[c]
4	903±84.42	633±54.44	70.08%± 1.01%[c]

Note: different lowercase letters indicate significant differences between groups ($p<0.05$).

3.2 Binding amount and binding rate of anti-*Salmonella* antibody to *Salmonella*

The numbers of *Salmonella* in the well was 2000-3500 CFU, and the *Salmonella* bound by anti-*Salmonella* antibody reached saturation at ~1700(Fig.2A). The binding capacity did not increase with the the numbers of bacteria added in the well, but showed a stable binding capacity. On the premise that there is a certain binding amount of *Salmonella* and anti-*Salmonella* antibody, it is necessary to further clarify the binding efficiency of *Salmonella* and anti-*Salmonella* antibody. Therefore, we reduce the numbers of *Salmonella* in the well to reduce the systematic error. When the numbers of *Salmonella* in the well lower than the maximum binding amount of *Salmonella*, the binding rate can't up to 100%, the numbers of salmonella bound with anti-*Salmonella* was floating, and the binding rate was stable at about 70% (Fig.2B).

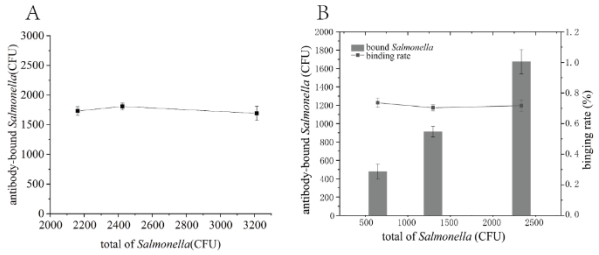

Figure 2. *Salmonella* binding capacity (A) and binding rate (B) with anti-*Salmonella* antibody

3.3 Construction of *Salmonella*-absorbance standard curve by BA-ELISA

The construction of *Salmonella* -absorbance standard curve for quantitative cytosolic *Salmonella* is essential for the isolation of vacuolar *Salmonella* and cytosolic *Salmonella* by BA-ELISA. The anti-*Salmonella* antibody in the well binds to the cytosolic *Salmonella*, while the SCV does not bind to the anti-*Salmonella* antibody, so it is necessary to quantify the unbound *Salmonella* by plate counting. The *Salmonella* bound antibody can be quantified by OD_{450nm} value (Fig.3A and Fig.3B). There is no linear and exponential relationship between numbers of *Salmonella* and OD_{450nm} value, but there is a linear positive correlation between $lg^{(number\ of\ Salmonella)}$ and $lg^{OD450nm}$, which can quickly and accurately calculate the number of *Salmonella* in the well.

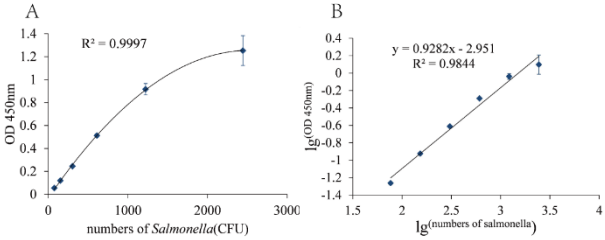

Figure 3. Construction of *Salmonella*-Absorbance Standard Curve by BA-ELISA

3.4 Isolation of intracellular cytosolic *Salmonella* and vacuolar *Salmonella*

The total of intracellular bacteria, cytosolic *Salmonella* and vacuolar *Salmonella* were counted by BA-ELISA and flat colony counting, the total of intracellular bacteria proliferated slowly within 4h of *Salmonella* infection (Fig.4A). In the early stage of infection, most *Salmonella* survived in SCV and proliferated at a moderate rate or survived in a dormant state[14]. The proportion of numbers of vacuolar *Salmonella* was negatively correlated during *Salmonella* infection, resulting in only about 23% of vacuolar *Salmonella* (16h). The proliferation rate was significantly lower than cytosolic *Salmonella*. With the prolongation of infection time, the *Salmonella* escaped from SCV and propagated rapidly in the cytoplasm. The numbers of cytosolic *Salmonella* in the cytoplasm exceeded the numbers of vacuolar *Salmonella* at 8h after *Salmonella* infection, and far exceeded the vacuolar *Salmonella* at 16h, and the proportion in the cell gradually increased (Fig.4B), the proportion of cytosolic *Salmonella* and vacuolar *Salmonella* was about 22% and 70% after

Salmonella infection (2h), and the proportion of cytosolic *Salmonella* in the host cell was positively correlated during *Salmonella* infection, reaching about 65% (16h).

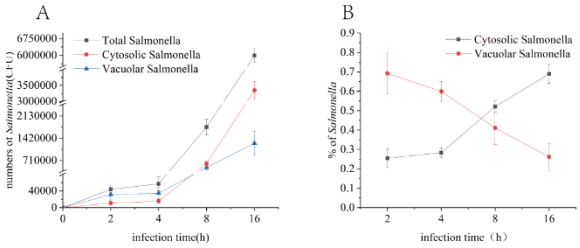

Figure 4. A Dynamic growth curve of *Salmonella* in Henle-407 cells; B Relative percentage of cytosolic *Salmonella* and vacuolar *Salmonella* in Henle-407 cells

4 Conclusion

Salmonella enterica serovar Typhimurium can inject effector proteins into host cells via type III secretion systems (T3SSs), the effector induces the recombination of cytoskeleton proteins and formation of ruffles in the host cell membrane, establishing a replicative niche termed SCV[15-16], which permits *S.* Typhimurium survive and reproduce within SCV. Meanwhile, part of Salmonella escape from SCV leading to the robust replication within cytosol, cytosolic *Salmonella* and vacuolar *Salmonella* coexisted in the host cells[17]. But there are significant differences in intracellular sublocalization and reproduction rate between the two forms of *Salmonella*, the current researches on the mechanism of *Salmonella* infection are mostly based on the total numbers, and there is no clear distinction between cytosolic *Salmonella* and vacuolar *Salmonella*.

In this study, we use BA-ELISA combined with flat colony counting methods to isolate and quantify intracellular cytosolic *Salmonella* and vacuolar *Salmonella*, so as to obtain a more intuitive reproductive trend of cytosolic *Salmonella* and vacuolar *Salmonella*. First, we determined the antibody concentration and antigen-antibody binding time. Second, through the antigen-antibody specific binding based on BA-ELISA, we confirmed the quantitative binding of anti-*Salmonella* antibody to about 1700 of *Salmonella*. Unlike protein, *Salmonella* is always in a state of movement within buffer. Although under the influence of gravity , *Salmonella* can bind to antibody , this effect has a certain relationship with time and temperature, resulting in that the binding rate between *Salmonella* and antibody is only 70%. Third, intracellular *Salmonella* was obtained by breaking cells in a homogenizer. With the help of homogenized buffer, this mild fragmentation method not only released intracellular *Salmonella* but also minimized the damage to SCV. Based on the binding capacity and binding rate, the numbers of cytosolic *Salmonella* were quantified by BA-ELISA, so far, the vacuolar *Salmonella* and cytosolic *Salmonella* were isolated and quantified in a direct and clear way. It is more intuitive and accurate to obtain the dynamic reproduction trend of two forms of *Salmonella* in the host cells. This method improves the counting way of intracellular *Salmonella* and provides a theoretical basis for the study of the mechanism of *Salmonella* infection.

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