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Screening of infection conditions for brain microvascular endothelial cells infected by *Streptococcus suis*

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Abstract

Streptococcus suis is a pathogen that causes swine meningitis, sepsis, and other diseases. There are 34 serotypes, of which type 2 is the most pathogenic. During the infection process of *Streptococcus suis*, several major virulence factors are involved and play a different roles. *Streptococcal* meningitis is caused by the bacteria's ability to cross the blood-brain barrier and enter the central nervous system. Therefore, studying the interaction between *Streptococcus suis* and cerebral microvascular endothelial cells will help reveal meningitis's pathogenic mechanism. When studying the interaction between bacteria and cells, the number of infected bacteria and the time of infection are very important. In this study, *Streptococcus suis* serotype two was made into bacteria liquid and counted. Then bacteria were used to infect mouse brain microvascular endothelial cells with different multiplicity of infection (1,10,100 and 200). Cells were harvested at six h, nine h,12h,18h, and 24h after infection. The total RNAs of harvested cells were extracted, and the concentration of RNA was detected. The OD260/OD280 was between 1.8~2.4, OD260/OD230 was 1.5~2.4, and the concentration was greater than 100ng/μL. Total RNAs were reverse transcribed to cDNAs used to perform quantitative PCR to detect the mRNA expression of IL-18, IL-1beta, IL-6, and IL-10. The results showed that each MOI group's mRNA expression is higher than the control group with different infection times. When the multiplicity of infection is at 1, each group's relative expression of cytokines reaches a peak at 18hrs after infection. When the multiplicity of infection is at 10, each group's relative expression of cytokines reaches a peak at 12hrs after infection. When the multiplicity of infection is at 100, the relative expression of cytokines reaches a peak at 12hrs after infection. When the multiplicity of infection is at 200, the relative expressions of each cytokine reach a peak at 6hrs after infection. Based on the mRNA relative expression of each cytokine under different conditions, the optimal multiplicity of infection was 100, and the optimal infection time was 12h. The result provides a basis for the study of the pathogenic mechanism of meningitis.

Keywords: screen, *Streptococcus suis 2*, infection, mouse brain microvascular endothelial cells.

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1. Introduction

Streptococcus suis (S. suis), an important zoonotic pathogen, can cause a variety of diseases in pigs, usually characterized by sepsis, meningitis, arthritis, lymphadenitis, and can also cause human meningitis, sepsis, etc., a serious threat pig industry and human health (Bağcigil et al., 2013; Gottschalk & Segura, 2019; Li et al., 2019). According to the capsular polysaccharide antigen (CPS) of bacteria, it can be divided into 35 serotypes, namely type 1 to type 34 and type 1/2 (strains containing both type 1 and type 2 antigens) (Okura et al., 2013). Different serotypes of *S. suis* and strains of the same type have different pathogenicity. *S.suis*2 is generally considered to be the most virulent and most prevalent *S. suis* strain (Auger et al., 2016; Schultsz et al., 2012; Segura, 2020).

The virulence factors currently identified in *S. suis* mainly include capsular polysaccharide(CPS), suilysin (SLY), extracellular factor (EF) and muramidase— released protein (MRP), and so on (Gottschalk et al., 2013). These virulence factors play different roles during infection.

S. suis is first determined in the mucous membranes of pigs' respiratory and gastrointestinal tract. During this process, MRP is processed by sortase A (SrtA), anchored on the cell wall peptidogly, and then combined with ECM, which is involved in the adhesion of bacteria (Bai et al., 2020). Under the action of force factor, it invades the blood and proliferates in the blood to form bacteremia. In this process, Sly is a cholesterol-binding cytotoxin that plays a role in the process of *S. suis* invading and lysing cells (Tenenbaum et al., 2016). It can destroy microvascular endothelial cells, epithelial cells, and macrophages, which is conducive to the spread of bacteria in tissues (Lecours et al., 2011). At the same time, *S. suis* has to resist the phagocytosis ofd macrophages. *S. suis* mainly relies on CPS to resist the phagocytosis of macrophages. CPS can prevent *S. suis* from being phagocytosed and killed by neutrophils and mononuclear macrophages (Korir et al., 2017; Yanyan Zhang et al.,

2016). Bacteria cross the blood-brain barrier to invade the meninges and central nervous system, causing meningitis.

S. suis causes meningitis, and the key process is that Streptococcus must cross the blood-brain barrier (BBB) and enter the central nervous system (CNS). The main component of the BBB is brain microvascular endothelial cells (BMEC) (Lim et al., 2017). BMEC can form tight junctions and express transport proteins, protect the CNS from the invasion of microorganisms and toxic substances in the blood, and maintain the stability of the CNS microenvironment (Yan et al., 2021).

In this study, mice were used as animal models to study the interaction between *S. suis* and mouse brain microvascular endothelial cells (bEnd.3). The screening of optimal multiplicity of infection and optimal infection time can provide experimental conditions for the study of the mechanism of meningitis.

2. Materials and methods

Preparation of bacterial solution

S. suis 2 was taken out from a -80 refrigerator, and 100 microliters were inoculated in THB liquid medium and shaken in a shaker at 37 degrees overnight. Bacteria were counted by the plate count method. Then 10 ml of bacterial liquid was added into ten centrifuge tubes separately, and the tubes were centrifuged at 3000 rpm for 5 minutes. The supernatants were discarded, and 10 ml sterile PBS was added into tubes separately and suspended bacteria. The bacteria liquid was centrifuged at 3000 rpm for 5 minutes. The supernatants were discarded, and 10 ml DMEM culture media without fetal bovine serum (FBS) were added into tubes and suspended them.

Cell culture

When bEnd. Three cells are in a logarithmic growth phase; cells were plated in a 6-well plate at a density of 1.0×10^6 cells/well and cultured at 37°C, 5% CO_2 for 12 hours. When the cell density reached 80 %~90 %, *S.suis2* was added into the well respectively with 1.4μL, 14μL, 140 μL, and 280 μL; the corresponding multiplicity of infection (MOI) are 1, 10, 100, and 200 respectively. The plates were cultured continuously in the incubator.

Detection of qPCR

According to the different culture times, the medium was discarded from the 6-well plate respectively after 6hs, 9hs, 12hs, 18hs, and 24hrs. The plates were washed twice with PBS, and 1 mL of Trizol was added to each well to extract total cell RNA according to the instructions. Nanodrop 2000 was used to detect the concentration and purity of RNA to make sure the values of OD260/OD280 are between 1.8~2.4, OD260/OD230 are 1.5~2.4, and the concentration is greater than 100ng/μL. The total RNA was reverse transcribed to obtain cDNA using a reverse transcription kit, and the one-step method was used to detect IL-18 mRNA, IL-1beta mRNA, IL-6 mRNA, and IL-10 mRNA. The upstream primer sequence of IL-18 is GACTCTTGCGTCAACTTCAAGG, and the downstream primer sequence of IL-18 is CAGGCTGTCTTTTGTCAACGA. The length of the target fragment is 169 bp. The upstream primer sequence of IL-1beta is TTCAGGCAGGCAGTATCACTC, and the downstream primer sequence of IL-1beta is GAAGGTCCACGGGAAAGACAC. The length of the target fragment is 75 bp. The upstream primer sequence of IL-6 is AGTTGCCTTCTTGGGACTGA, and the downstream primer sequence of IL-6 is TCCACGATTTCCCAGAGAAC. The target fragment length is 159 bp. The upstream primer sequence of IL-10 is CTGCTATGCTGCCTGCTCTTACTG, and the downstream primer sequence of IL-10 is ATGTGGCTCTGGCCGACTGG. The target fragment length is 104 bp. The upstream primer sequence of GAPDH is AGGTCGGTGTGAACGGATTTG, and the downstream primer sequence is TGTAGACCATGTAGTTGAGGTCA. The length of the target fragment is 123 bp.

qPCR was performed using SYBR@ Premix Ex Taq™ II（Takara Bio Inc. Dalian, RR820A）by Q5 detection system (ABI). All reaction was carried out in a 20μL reaction volume. Amplification conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C and 34 s at 60 °C. Each sample was set with three parallel replicate wells. Using GAPDH as the internal reference gene, the relative expression of the target gene was calculated according to the $2^{-\Delta\Delta Ct}$ method.

3. Results and discussion

The relative expression of cytokines in each MOI group is all higher than the control group with different infection times. When MOI is at 1, each group's relative expression of cytokines reaches a peak at 18hrs after infection (Fig 1). When MOI is at 10, each group's relative expression of cytokines reaches a peak at 12hrs after infection (Fig 2). When MOI is at 100, the relative expression of cytokines reaches a peak at 12hrs after infection (Fig 3). When MOI is at 200, the relative expressions of each cytokine reach a peak at 6hrs after infection (Fig 4).

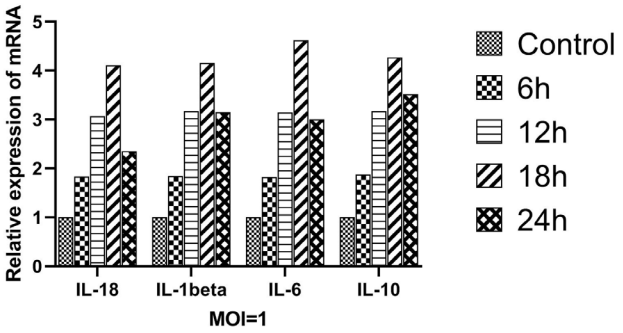

Fig. 1. Effect of *S.suis2* infection on the expression of cytokines (MOI = 1)

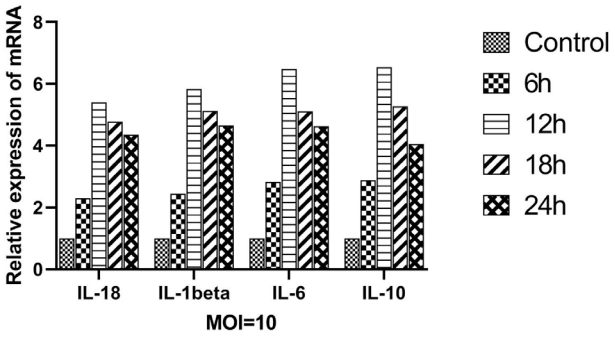

Fig. 2. Effect of *S. suis 2* infection on the expression of cytokines (MOI = 10)

Based on the mRNA relative expression of each cytokine under different conditions, when the MOI=100 and the infection time was 12h, the mRNA expression of each cytokine was the highest.

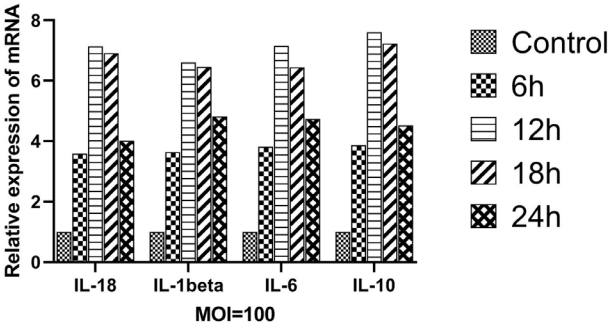

Fig. 3. Effect of *S. suis 2* infection on the expression of cytokines (MOI = 100)

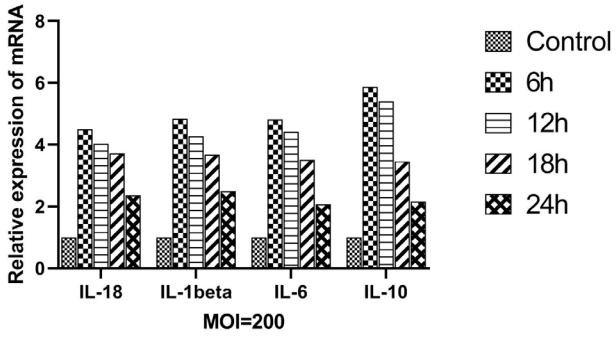

Fig. 4. Effect of *S.suis2* infection on the expression of cytokines (MOI = 200)

The plate counting method is often used to calculate bacteria. The method includes plate mixed culture and spread plate method. Mixed plate culture needs to mix bacteria liquid and agar medium when the temperature of the agar medium is about 40 degrees (Gabriel-Ajobiewe, 2011). It is easy to affect the activity of bacteria when the temperature is not controlled well, and then the density of bacteria is affected subsequently (Yang et al., 2018). The spread plate method puts bacteria liquid after the agar medium is cold and solidifies. It avoided the shortage of mixed plate culture and was used in this study (Arangannal et al., 2019).

Meningitis is a common disease that restricts the pig industry's development. *S. suis 2* infection is the main pathogenic factor of meningitis (Hlebowicz et al., 2019). Exploring the relationship between Streptococcus and meningitis is an important basis for preventing and treating the disease. Numerous studies have shown a link between streptococcal infection and apoptosis (Rai et al., 2015; D. Xu et al., 2017). As another way of cell death, Is pyroptosis involved in the occurrence of meningitis? The main features of pyroptosis are cell enlargement, cell membrane rupture, and release of cell contents represented by IL-18 and IL-1beta (Yingying Zhang et al., 2018). As inflammatory factors, IL-6 and IL-10 are normally present in very low levels in the body. When microorganisms infect the body, the immune system is activated, and the contents of IL-6 and IL-10 will increase (An et al., 2018). High levels are positively correlated with inflammation. The more severe the inflammation, the higher the contents are. Especially in bacterial infections, the elevated levels of IL-6 and IL-10 were significantly higher than those in non-bacterial infections (Xu et al., 2012).

The results showed that the mRNA expression of the corresponding cytokines increased with the increase of MOI value when bEnd.3 was infected with different MOI. When MOI is at 1, due to the small number of infected Streptococcus, the mRNA expression of cytokines in each group was not significantly increased compared with the control group and reached a peak at 18h. When MOI is at 10, the mRNA expression of cytokines in each group increases relative to MOI at one and reaches a peak at 12 h. With the increase of *S. suis 2*, the expression of cytokines in each group increased significantly when MOI was at 100, compared with other infection times, and 12 h and 18h were relatively more expressed. However, when the MOI was at 200, the mRNA expression of cytokines in each group decreased again, mainly because the infection time was too long, a large number of cells died, and they were lysed into cell fragments.

4. Conclusions

In this study, *S. suis 2* was multiplied and calculated. The bacteria liquid was used to infect bEnd.3 with different MOIs and infection times. QPCR was used to detect the mRNA expression of cytokines under different conditions. The optimal MOI is 100, and the optimal infection time is 12 h. The results can provide a basis for the pathogenic mechanism of meningitis.

Conflict of interest

The authors declare that there is no conflict of interest.

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