Quantitative assessment of the blood-brain barrier opening caused by *Streptococcus agalactiae* hyaluronidase in a BALB/c mouse model

Su Luo, Qing Cao, Ke Ma, Zhaofei Wang, Guangjin Liu, Chengping Lu & Yongjie Liu

*Streptococcus agalactiae* is a pathogen causing meningitis in animals and humans. However, little is known about the entry of *S. agalactiae* into brain tissue. In this study, we developed a BALB/c mouse model based on the intravenous injection of β-galactosidase-positive *Escherichia coli* M5 as an indicator of blood-brain barrier (BBB) opening. Under physiological conditions, the BBB is impermeable to *E. coli* M5. In pathological conditions caused by *S. agalactiae*, *E. coli* M5 is capable of penetrating the brain through a disrupted BBB. The level of BBB opening can be assessed by quantitative measurement of *E. coli* M5 loads per gram of brain tissue. Further, we used the model to evaluate the role of *S. agalactiae* hyaluronidase in BBB opening. The inactivation of *hylB* gene encoding a hyaluronidase, HylB, resulted in significantly decreased *E. coli* M5 colonization, and the intravenous injection of purified HylB protein induced BBB opening in a dose-dependent manner. This finding verified the direct role of HylB in BBB invasion and traversal, and further demonstrated the practicability of the *in vivo* mouse model established in this study. This model will help to understand the *S. agalactiae*–host interactions that are involved in this bacterial traversal of the BBB and to develop efficacious strategies to prevent central nervous system infections.

*Streptococcus agalactiae*, also known as Group B *Streptococcus* (GBS), is a Gram-positive, opportunistic pathogen that colonizes the gastrointestinal and genitourinary tracts of up to 50% of healthy adults. It also causes sepsis, meningitis and pneumonia in neonates and is the main reason for significant morbidity in pregnant women, the elderly and immunocompromised adults. *S. agalactiae* can also infect the mammary glands of cows, where it can survive for a long period of time, causing clinical or subclinical mastitis. In addition, in recent years, it has been reported that *S. agalactiae* can cause meningoecephalitis in fish and bring losses to aquaculture. Numerous outbreaks of *S. agalactiae* infections have been described in multiple fish farms, especially tilapia farms. Since 2009, an outbreak of severe infectious GBS disease has occurred in tilapia farms in the south of China, causing tremendous economic losses due to high mortality in the infected fish.

The pathogenesis of meningitis caused by *S. agalactiae* has not been fully elucidated. It is well known that only pathogens entering the central nervous system (CNS) can cause meningitis. To gain access into the CNS, *S. agalactiae* must cross the blood-brain barrier (BBB), which is primarily comprised of a single layer of specialized brain microvascular endothelial cells (BMECs). This unique brain endothelial physiological barrier seals the CNS, and regulates passage of molecules, nutrients, and infectious agents into the brain. Pathogens can cross the BBB transcellularly, paracellularly and by a “Trojan-horse” mechanism. Some studies have demonstrated that *S. agalactiae* virulence factors contribute to the adherence and invasion of human BMECs (hBMECs), resulting in the activation of acute inflammatory responses that inevitably disrupt the integrity of the BBB. For example, the well-known virulence factor β-hemolysin/cytolysin stimulated the expression of genes linked to neutrophil recruitment and activation, such as IL-8 and ICAM-1, which act to facilitate neutrophil migration across polar hBMEC monolayers. PilA, the pilus tip adhesin of GBS, had capability in promoting the expression of
neutrophil chemokines IL-8, CXCL-1, CXCL-2, CCL-20 and IL-6 in brain endothelium, and therefore increased
the permeability of the BBB\textsuperscript{19}. Several investigations in animals have also shown that GBS can penetrate the CNS
by crossing the BBB after a prolonged period of bacteremia\textsuperscript{20,21}. Therefore, the BBB plays an important role in
controlling the entry of pathogens into the brain.

Increased permeability of the BBB can be seen in bacterial meningitis caused by \textit{S. agalactiae}\textsuperscript{22,23}. However,
how this bacterium crosses the BBB and enters the CNS is not clearly understood. Therefore, it is of crucial
importance to characterize BBB permeability in order to better understand the pathogenesis of meningitis. In our
previous study, the inactivation of \textit{hylB} gene encoding a hyaluronidase, HylB, caused the significantly decreased
brain bacterial counts in mice\textsuperscript{24}. However, whether HylB directly acts on the BBB opening remains unclear. In this
study, we sought to establish a model to evaluate BBB opening by screening a bacterial strain as an indicator, and
used this model to quantitatively evaluate the direct role of HylB in \textit{S. agalactiae} penetration across BBB.

Results

The screening of an indicator strain. To make the counting easy, we aimed to find a bacterium with
a colony morphology distinct from that of \textit{S. agalactiae}. The results showed that among the M1 to M5 \textit{E. coli}
mutants, only the M5 strain generated characteristic blue clones on M63 media (Fig. 1), indicating that it was
β-galactosidase-positive.

Determination of \textit{E. coli} M5 virulence in mice. To determine whether \textit{E. coli} M5 was virulent to mice,
we performed bacterial infection in BALB/c mice. Interestingly, it was observed that none of the mice infected
with $2 \times 10^9$ CFU of \textit{E. coli} M5 showed any signs of illness, and there was zero mortality throughout the exper-
imental period of 7 d. The diet and mental state of the experimental mice were identical to those of the control
group. The result indicated that \textit{E. coli} M5 was avirulent to mice.

Kinetics of \textit{E. coli} presence in blood of mice. To investigate the rate of \textit{E. coli} M5 metabolism in mice,
the mice were injected intravenously with M5, and blood samples were collected from each mouse at intervals of 3 min, 5 min, 10 min, 30 min, 60 min and 120 min post-infection. The CFU enumeration results showed
that the number of M5 cells in the blood increased from $6 \times 10^5$ CFU/mL at 3 min to a peak value of approximately
$2.5 \times 10^6$ CFU/mL at 5 min and then decreased dramatically to $3 \times 10^5$ CFU/mL at 10 min. After one hour,
bacteria could hardly be detected, and they had clearly been removed from circulation within 2 hours after intravenous injection (Fig. 2). No bacteria were detected in the brain at any time points.

Determination of a challenge concentration of \textit{S. agalactiae}. We first sought to determine whether the \textit{S. agalactiae} GD201008-001 strain causes significant BBB permeability by intraperitoneal infection. It is important to determine an appropriate initial inoculation concentration for \textit{S. agalactiae} to create an \textit{in vivo} model. We chose values of 5-fold (50 CFU) and 10-fold (100 CFU) that of the median lethal dose (LD\textsubscript{50} < 10 CFU)\textsuperscript{25} of \textit{S. agalactiae} to inoculate the mice. The result showed that with the duration of infection, the number of GD201008-001 cells increased in both the blood (Fig. 3A) and the brain (Fig. 3B), suggesting that GD201008-001 was capable of replicating within the bloodstream and spreading to the brain. GD201008-001 began to appear in the blood at 3 h post-infection, 3 h earlier than in the brain. The bacteria could be detected in the brain at 6 h post-infection when we injected 100 CFU of \textit{S. agalactiae}. However, we could not detect any
bacteria until 12 h post-infection in the 50 CFU group. This result suggests that the intraperitoneal infection with 100 CFU of *S. agalactiae* is more appropriate to induce BBB opening in this mouse model.

**Evaluation of BBB opening.** Groups of five mice were challenged with 100 CFU of GD201008-001. At 3 h, 6 h, 9 h and 12 h after challenge, the indicator strain *E. coli* M5 was inoculated intravenously into each group. In determining the sampling time points, based on Fig. 2, more M5 tracers were detected in the blood at 5 min. Therefore, 5 min after injection was selected to quantify the degree of BBB permeability. Our data showed that GD201008-001 began to appear in the brain 6 h post-infection, and with the duration of infection, the bacterial number increased (Fig. 4). M5 could be detected 3 h after *S. agalactiae* inoculation, and the number of M5 had a similar increasing trend as GD201008-001 (Fig. 5). This result suggests that *E. coli* M5 may be used as an appropriate indicator for describing the degree of BBB opening.

**Detection of BBB opening caused by *S. agalactiae* hyaluronidase.** Previous study showed that hyaluronidase contributed to *S. agalactiae* penetration into the CNS. To verify the utility of this mouse model, the mice were infected with the wild-type *S. agalactiae*, ΔhylB and CΔhylB strains, and then *E. coli* M5 was administered to the mice. After intraperitoneal infection with 100 CFU of *S. agalactiae* and its derivatives, the numbers of the three bacterial strains in the blood increased rapidly from <10³ CFU/mL at 3 h to >10⁷ CFU/mL at 15 h (Fig. 6A). In the brains, the wild-type and the complemented strain CΔhylB were present 6 h post-infection, which was earlier than the mutant ΔhylB (Fig. 6B). Compared with the wild-type and complemented strains, for the hylB mutant, the numbers were lower at each time point in the brains. As time passed, the number of bacteria in the brains increased to >10⁵ CFU/g at 15 h post-infection. *E. coli* M5 in the wild-type and CΔhylB groups could be detected in the brain tissues 6 h post-infection, and the increasing trend of the number of M5 cells was similar to that of *S. agalactiae* (Fig. 6C). The sudden increase in the number of M5 at 15 h indicated that the BBB of the mice had opened to a great degree. However, in the ΔhylB group, the ΔhylB strain and M5 were not detected until 9 h post-infection, and the amount of M5 was significantly lower than that of the wild-type group at 9 h post-infection (*P* < 0.001). At 12 h and 15 h, the difference in the number of M5 cells between the wild-type and ΔhylB groups reached a highly significant difference (*P* < 0.001). Although the number of M5 cells in the CΔhylB group was lower than that in the wild-type group, the difference was smaller than that with the ΔhylB group.

To further investigate the role of the hylB gene in BBB opening, the protein HylB, which is encoded by the hylB gene, was expressed and injected intravenously into the mice. An injection of 0.5 mg/mL HylB did not cause BBB opening.
opening at 3 h, as evidenced by the absence of *E. coli* M5 in brain, whereas M5 could enter and begin to accumulate in the brains of the 1.0 mg/mL and 2.0 mg/mL groups (Fig. 7). There was a dose-dependent increase in the degree of BBB opening at each time point.

**Discussion**

Meningitis is the most common clinical syndrome of *S. agalactiae* infection. Bacterial penetration across the BBB and into the CNS is the first step in the development of meningitis. Therefore, adequate BBB models need to be developed in order to characterize the properties of bacterial penetration into the CNS. The *in vitro* BBB model based on the culture of brain microvascular endothelial cells has been widely used to probe the potential role(s) of individual virulence determinants in the initial pathogenesis of CNS infection by *S. agalactiae*. For example, hBMEC has been used to determine the invasive roles of fibronectin binding protein A (SfbA), laminin-binding protein (Lmb) and the surface protein HvgA in GBS infection. However, the *in vitro* model might not completely mimic the disease in animals or humans. It was reported that CovR-deficient GBS showed a decreased ability to invade the brain endothelium *in vitro*, but *in vivo*, this deletion mutant was more proficient in the induction of permeability and proinflammatory signaling pathways in the brain endothelium and in penetration of the BBB. In contrast, a previous study on the major pilin subunit PilB reported that the *pilB* mutant was less
In considering the dose of HylB protein, we tested a treatment of 3 mg/mL HylB, but all the mice died 15 h earlier than in the 0.5 mg/mL group, and the number of virulent bacteria increased with the time of infection. The data are expressed as CFU per gram of brain.

Figure 7. Evaluation of BBB opening in mice injected with different doses of hyaluronidase. Groups of four BALB/c mice were inoculated with 0.5, 1.0 or 2.0 mg/ml of HylB, and at different time points post-infection, E. coli M5 (2 × 10⁶ CFU) was injected intravenously. Five minutes later, the mice were killed, and the brains were removed for quantification of M5. The data are expressed as CFU per gram of brain. *P < 0.05, **P < 0.01 or ***P < 0.001 indicates significantly different bacterial loads between the two infection groups.

The mouse has been widely used for investigating S. agalactiae virulence in recent years. In our study, the parameters and criteria may be variable due to different bacterial species. In our study, this model was optimized for use in S. agalactiae, and demonstrated to be a powerful method for analyzing the BBB opening.

Under physiological conditions, the BBB strictly regulates the entry of blood-borne substances into the brain. Brain inflammation can affect the permeability of the BBB directly via cytokine-mediated activation of metalloproteases or tight junction disruption, or indirectly by promoting transmigration of leukocytes. In S. agalactiae, hyaluronidase has been demonstrated to contribute to the bacterial invasion and the pathogenesis of meningitis in mice. However, unlike the PilA and β-hemolysin/cytolysin which stimulate the release of pro-inflammatory cytokines, hyaluronidase acts as an anti-inflammation factor instead. Our previous study demonstrated that compared to the wild-type S. agalactiae, the hyaluronidase-deficient mutant stimulated a significantly higher level of pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α in macrophages, whereas its mortality to zebrafish was lower. Afterwards, a probable reason for this phenomenon was illustrated by Kolar et al.

They revealed that GBS evades host immunity by degrading hyaluronan (HA) which is a component of extracellular matrix nearly in all tissues. HA is commonly cleaved into small fragments by tissue hyaluronidase in response to tissue injury. These small HA fragments are inflammatory factors that ligate to Toll-like receptor (TLRs) to elicit inflammatory response and repair the damaged tissue. However, bacterial hyaluronidase degrades pro-inflammatory HA fragments to the major end product disaccharides. HA disaccharides bind to TLR2/4 to block signaling elicited by host HA fragments and other TLR2/4 ligands, thus preventing GBS ligands from activating pro-inflammatory signaling cascades. Therefore we assume that hyaluronidase contributes to GBS meningitis by anti-inflammation and evasion of host immune. However, our recent study showed that intravenous injection of a purified hyaluronidase, HylB, induced acute lung and brain injury.

This led us to speculate that HylB might play important role in BBB permeability. In order to evaluate this speculation, we first investigated the role of HylB in disrupting the BBB integrity using this model established in this study. Compared with the wild-type S. agalactiae, the inactivation of hylB resulted in decreased BBB opening throughout the infection. Although the presence of S. agalactiae in brain indicates the BBB opening, the use of E. coli M5 as an indicator excludes the possibility that the differential BBB integrity may be caused by different proliferation abilities in vivo between the wild-type and hylB mutant strains.

To further determine whether HylB has a direct impact on BBB integrity, we intravenously injected the purified HylB protein into the mice. We found that the intravenous injection of HylB induced BBB opening in a dose-dependent manner. In the groups treated with 1 mg/mL and 2 mg/mL, the BBB was open 3 h post-infection, 3 h earlier than in the 0.5 mg/mL group, and the number of E. coli M5 increased with the time of infection. In considering the dose of HylB protein, we tested a treatment of 3 mg/mL HylB, but all the mice died 15 h
post-infection. This finding indicated that HylB is one of the important virulence factors of *S. agalactiae*, which is in agreement with previous studies. A similar role for hyaluronidase in inducing pneumococcal meningitis has also been reported by Zwijsenborg et al. The present investigation of HylB further demonstrates that using *E. coli* M5 as an indicator is an easy and reliable method for assessing BBB integrity and/or leakiness. In particular, the model could be more suitable to investigate the contribution of soluble bacterial virulence factors to BBB disruption.

In this study, we used a piscine strain of *S. agalactiae* with an extremely high virulence to BALB/c mice. It is not clear why this piscine strain is so virulent and what genetic relationship exists between fish and human isolates. Our previous study has made a comparative genomic analysis among 15 *S. agalactiae* strains of different origins, and found that the Chinese piscine isolates GD201008-001 and ZQ0910 are phylogenetically distinct from the Latin American piscine isolates SA20-06 and STIR-CD-17, but are closely related to the human strain A909. Additionally, a published study reported that a GBS isolate from a clinical case of human neonatal meningitis caused disease and death in Nile tilapia. In this regard, it may be of interest to further investigate the pathogenic mechanisms of meningitis caused by different origins of *S. agalactiae* strains. This model established here could be a potentially useful tool for the investigation. Nevertheless, it will be imperative to demonstrate that the *E. coli* tracer works with other GBS and mouse strains that are widely used in the meningitis model.

In summary, the present study developed a model that can quantify the degree of BBB opening caused by *S. agalactiae*, and used this model to demonstrate that hyaluronidase plays a direct role in BBB permeability.

**Methods**

**Bacterial strains and growth conditions.** *S. agalactiae* strain GD201008-001, β-hemolysin/cytolysin positive, which belongs to serotype Ia, MLST type ST-7, was isolated from farmed tilapia with meningoencephalitis in Guangdong Province, China, in 2010. Its genome sequence has been deposited in the GenBank database under accession number CP003810. The *S. agalactiae* hylB deleted mutant strain ΔhylB and the complemented strain CΔhylB were constructed in the previous study. All of the bacterial strains were grown using either Todd-Hewitt broth (THB) or agar (THA) (Becton Dickinson, MD, USA) or sheep blood agar plates at 37°C.

**Screening for a β-galactosidase-positive *E. coli* strain.** To describe the time and degree of BBB opening, an indicator bacterial strain was needed. Inspired by blue-white selection, five strains of engineered *E. coli* (numbered from M1 to M5) were grown overnight in the dark at 37°C in M63 basic medium (13.6% (w/v) KH₂PO₄, 0.4% (w/v) KOH, 0.2% (w/v) (NH₄)₂SO₄, 0.1 mM FeSO₄, containing 1 mM MgSO₄, 0.2% (w/v) lactose, 0.002% (w/v) 5-bromo-4-chloro-3-indolyl-L-D-galactopyranoside (X-gal) and 0.002% (w/v) vitamin B₁. The bacterial strains were screened for their ability to form blue colonies on M63 plates for use as an indicator in the study.

**Determination of *E. coli* M5 virulence in mice.** BALB/c mice (24–26 g, aged 5–6 weeks) were bought from the Experimental Animal Center, Yangzhou University. The mice were divided into two groups with 10 mice for each group. The screened *E. coli* M5 were grown overnight in LB broth. A bacterial suspension of 50 μL was transferred into 5 mL LB and incubated at 37°C to allow the cells to reach mid-log phase growth. When the bacteria reached an OD₆₀₀ of 0.6, they were harvested by centrifugation at 5000 × g for 5 min. The cell pellets were washed twice with sterile phosphate-buffered saline (PBS) (pH 7.4) and re-suspended in PBS to a concentration of 2 × 10⁷ CFU/mL. One group of mice was injected intravenously with 100 μL of bacterial suspension, whereas the other was injected with 100 μL PBS and served as a control. The mice were observed until one week post-infection.

**Kinetics of *E. coli* presence in blood of mice.** As an indicator, *E. coli* M5 should be eliminated rapidly from the circulatory system. Based on five predetermined time points, BALB/c mice (24–26 g, aged 5–6 weeks) were divided into five groups with eight mice for each group. Mid-log phase bacteria were washed twice with PBS, followed by re-suspension in PBS and adjustment of the concentration to 2 × 10⁹ CFU/mL. For each time point, five mice were used as the experimental group and were injected intravenously with 100 μL of bacterial suspension, while another three were injected intravenously with 100 μL of PBS. Blood samples and brains were obtained aseptically at 3 min, 5 min, 10 min, 30 min and 60 min post infection. Blood samples of 100 μL were spread onto M63 plates. To avoid surface contamination, the organs were washed twice with PBS. Tissues were placed in 1 mL of PBS and homogenized with a biological sample homogenizer (BioPrep-24, Ningbo Hinotek Instrument Co Ltd, China). Then, 100 μL of homogenate that was either undiluted or diluted 10⁻¹, 10⁻² and 10⁻³ in PBS were plated on M63 plates. The M63 plates were incubated overnight at 37°C. Colonies were counted and given as CFU/g for brain samples or CFU/mL for blood samples.

**Determination of the challenge concentration of *S. agalactiae*.** Our previous study has shown that the bacterial strain GD201008–001 is highly virulent to BALB/c mice by intraperitoneal administration, with LD₅₀ values of less than 10 CFU. Here, we chose two different doses of *S. agalactiae*, 50 and 100 CFU (5- and 10-fold greater than the LD₅₀), to find an applicable dose for this mouse model. BALB/c mice were divided into two groups with 16 mice for each group. One group received an intraperitoneal injection of 100 μL of 500 CFU/mL bacterial suspension, and the other received an injection of 1000 CFU/mL. In each group, four mice were sacrificed every three hours to aseptically collect the blood and brain. Homogenized brain tissues and blood were plated onto THB plates for bacterial cell counting to determine tissue colonization. The experiments were repeated at least three times to ensure reproducibility. The data are expressed as CFU/g or CFU/mL per mouse.
Evaluation of BBB opening. BALB/c mice were divided into five groups with five mice for each group. The mice were infected with a predetermined dose of 100 CFU of the strain GD201008-001. Control mice were injected with sterile PBS. Then, 2 × 10^8 CFU of the indicator E. coli M5 in 100 μL PBS was given intravenously at the specified time points (3 h, 6 h, 9 h and 12 h), and 5 min later, the mice were sacrificed. To detect the degree of BBB opening, the brains were removed aseptically and homogenized in PBS. The homogenate was serially diluted and spread onto THB or M63 agar plates, then incubated overnight at 37 °C. The organ CFU enumeration of S. agalactiae and E. coli M5 were determined and expressed as the mean and S.D. per mouse.

Detection of BBB opening caused by S. agalactiae hyaluronidase. To investigate the effect of BBB opening caused by S. agalactiae hyaluronidase, we used the deficient mutant strain ΔhylB and the complemented strain CΔhylB constructed in our previous study. One hundred and twenty mice were divided into three groups with 40 mice in each group. Mid-log phase S. agalactiae and its derivatives were washed twice in PBS and re-suspended in PBS to 1 × 10^8 CFU/mL. The concentration of E. coli M5 was adjusted to 2 × 10^9 CFU/mL. Three groups of mice were infected with 100 μL of the wild-type S. agalactiae, ΔhylB or CΔhylB by intraperitoneal injection. At 3 h, 6 h, 9 h, 12 h, and 15 h post infection, groups of four mice were killed, and the blood and brain tissues were collected for S. agalactiae quantification. Meanwhile, another four mice from each group were respectively inoculated with 100 μL of E. coli M5 by intravenous route at each time point as mentioned above. At 5 min post-inoculation with E. coli, the brains were aseptically removed and homogenized in PBS. The homogenates were serially diluted, spread onto THB plates for S. agalactiae counting or M63 plates for E. coli M5 counting and incubated overnight at 37 °C. The bacteria were counted and reported as CFU/g per mouse.

To further determine the role of the hylB gene in the pathogenesis of BBB opening caused by S. agalactiae, we expressed HylB with good enzymatic activity, as described in our previous study. Sixty mice were divided into three groups with 20 mice in each group. The three groups of the mice were intravenously injected with 200 μL of HylB protein at a final concentration of 0.5 mg/mL, 1.0 mg/mL and 2.0 mg/mL, respectively. Twenty control mice were injected with 200 μL of sterile PBS. Then, 100 μL of the E. coli M5 (2 × 10^8 CFU/mL) was inoculated intravenously at intervals of 3 h, 6 h, 9 h, 12 h and 15 h post infection, and the brain tissues were sampled at 5 min post-infection with E. coli. The homogenates were serially diluted, spread onto M63 plates and incubated overnight at 37 °C for E. coli M5 counting. The bacteria were counted and expressed as CFU/g per mouse.

Statistical analysis. Data were collected and analyzed using MS Excel 2010 and SPSS Statistics version 20.0 software. Multiple comparisons were performed by analysis of variance (ANOVA) followed by Turkey’s multiple-comparison test, with P < 0.05 indicating a statistically significant difference and P < 0.01 indicating a highly significant difference. The error bars presented in the figures represent the standard deviations of the means of multiple replicate experiments.

Experimental procedures

Ethics statement. All the animal experiments were carried out according to animal welfare standards and were approved by the Ethical Committee for Animal Experiments of Nanjing Agricultural University, China. All animal experiments complied with the guidelines of the Animal Welfare Council of China.

References

1. Hansen, S. M., Uldbjerg, N., Kilian, M. & Sorensen, U. B. S. Dynamics of Streptococcus agalactiae colonization in women during and after pregnancy and in their infants. J. Clin. Microbiol. 42, 83–89 (2004).
2. Dermer, P., Lee, C., Eggert, J. & Few, B. A history of neonatal group B streptococcus with its related morbidity and mortality rates in the United States. J. Pediatric. Nurs. 19, 357–363 (2004).
3. Rapoport, P. J. & Hofschire, P. J. Group B streptococcal infections in neonates. Nebr. Med. J. 41, 839–847 (1979).
4. Siddiqui, B. et al. The first documented case of hemorrhagic stroke caused by group B streptococcal meningitis. IDCases 2, 118–119 (2013).
5. Keefe, G. P. Streptococcus agalactiae mastitis: a review. Can. Vet. J. 38, 429–437 (1997).
6. Vandamme, P., Devriese, L. A., Pot, B., Kersters, K. & Melin, P. Streptococcus difficile is a nonhemolytic group B, type lb streptococcus. Int. J. Syst. Bacteriol. 47, 81–85 (1997).
7. Zunyi-Saad, M., Amal, M. N. A. & Sitia-Zahrah, A. Pathological changes in red tilapias (Oreochromis spp.) naturally infected by Streptococcus agalactiae. J. Com. Pathol. 143, 227–229 (2010).
8. Evans, J. J., Klesius, P. H., Pasnik, D. J. & Bohnsack, J. F. Human Streptococcus agalactiae isolate in Nile tilapia (Oreochromis niloticus). Emerg. Infect. Dis. 15, 774–776 (2009).
9. Amal, M. N. A. et al. An outbreak of Streptococcus agalactiae infection in cage-cultured golden pompano, Trachinotus blochii (Lacépède), in Malaysia. J. Fish Dis. 35, 849–852 (2012).
10. Chen, M. et al. PCR detection and PFGE genotype analyses of streptococcal clinical isolates from tilapia in China. Vet. Microbiol. 159, 526–530 (2012).
11. Saunders, N. R., Liddelow, S. A. & Dziugiela, K. M. Barrier mechanisms in the developing brain. Front. Pharmacol. 3, 1–18 (2012).
12. Segenhzeles, J. A., Sohet, E. & Daneman, R. ‘Sealing off the CNS’: cellular and molecular regulation of blood–brain barrier genesis. Curr. Opin. Neurobiol. 23, 1057–1064 (2013).
13. Armuluk, A. et al. Pericytes regulate the blood–brain barrier. Nature 468, 557–561 (2010).
14. Bell, R. D. et al. Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. Neuron 68, 409–427 (2010).
15. Daneman, K., Zhou, L., Kebese, A. A. & Barres, B. A. Pericytes are required for blood–brain barrier integrity during embryogenesis. Nature 468, 562–566 (2010).
16. Eigenmann, D. E. et al. Comparative study of four immortalized human brain capillary endothelial cell lines, hCMEC/D3, hBMSC, TY10, and BB19, and optimization of culture conditions, for an in vitro blood–brain barrier model for drug permeability studies. Fluids Barriers CNS 10, 33 (2013).
17. Kim, K. S. et al. Mechanism of microbial traversal of the blood–brain barrier. Nat. Rev. Microbiol. 6, 625–634 (2008).
18. Doran, K. S., Liu, G. Y. & Nizet, V. Group B streptococcal 3–hemolysin/cytolysin activates neutrophil signaling pathways in brain endothelium and contributes to development of meningitis. *J. Clin. Invest.* **112**, 736–744 (2003).
19. Banerjee, A. et al. Bacterial pili exploit integrin machinery to promote immune activation and efficient blood–brain barrier penetration. *Nat. Commun.* **2**, 462 (2011).
20. Doran, K. S. *et al.* Blood–brain barrier invasion by group B streptococcus depends upon proper cell-surface anchoring of lipoteichoic acid. *J. Clin. Invest.* **115**, 2499–2507 (2005).
21. Maisey, H. C., Doran, K. S. & Nizet, V. Recent advances in understanding the molecular basis of group B streptococcus virulence. *Expert Rev. Mol. Med.* **10**, e27 (2009).
22. Kim, K. S., Wass, C. A. & Cross, A. S. Blood–brain barrier permeability during the development of experimental bacterial meningitis in the rat. *Exp. Neurol.* **145**, 253–257 (1997).
23. Kim, B. J. *et al.* Bacterial induction of Snail1 contributes to blood–brain barrier disruption. *J. Clin. Invest.* **125**, 2473–2483 (2015).
24. Wang, Z. *et al.* Two novel functions of hyaluronidase from *Streptococcus agalactiae* are enhanced intracellular survival and inhibition of proinflammatory cytokine expression. *Infect. Immun.* **82**, 2615–2625 (2014).
25. Guo, C. M. *et al.* Identification of genes preferentially expressed by highly virulent piscine *Streptococcus agalactiae* upon interaction with macrophages. *PLoS One* **9**, e87980 (2014).
26. Tsao, N., Chang, W. W., Liu, C. C. & Lei, H. Y. Development of hematogenous pneumococcal meningitis in adult mice: the role of TNF-α. *FEMS Immunol. Med. Microbiol.* **32**, 133–140 (2002).
27. Mu, R. *et al.* Identification of a group B streptococcal fibronectin binding protein, SfbA, that contributes to invasion of brain endothelium and development of meningitis. *Infect. Immun.* **82**, 2276–2286 (2014).
28. Tenenbaum, T. *et al.* *Streptococcus agalactiae* invasion of human brain microvascular endothelial cells is promoted by the laminin-binding protein Lmb. *Microbes Infect.* **9**, 714–720 (2007).
29. Tazi, A. *et al.* The surface protein HvgA mediates group B streptococcus hypervirulence and meningeal tropism in neonates. *J. Exp. Med.* **207**, 2313–2322 (2010).
30. Lembo, A. *et al.* Regulation of CovR expression in group B streptococcus impacts blood–brain barrier penetration. *Mol. Microbiol.* **77**, 431–443 (2010).
31. Papasergi, S. *et al.* The GBS PI-2a plas is required for virulence in mice neonates. *PLoS One* **6**, (2011).
32. Patras, K. A. *et al.* Group B streptococcus CovR regulation modulates host immune signaling pathways to promote vaginal colonization. *Cell Microbiol.* **15**, 1154–1167 (2013).
33. Kaya, M. & Abishali, B. Assessment of permeability in barrier type of endothelium in brain using tracers: Evans blue, sodium fluorescein, and horseradish peroxidase. *Methods Mol. Biol.* **763**, 369–382 (2011).
34. Radu, M. & Chernoff, J. An in vivo assay to test blood vessel permeability. *J. Vis. Exp.* **73**, e50862 (2013).
35. Saunders, N. R., Dziegielewksa, K. M., Møllgård, K. & Habgood, M. D. Markers for blood–brain barrier integrity: how appropriate is Evans blue in the twenty-first century and what are the alternatives? *Front. Neurosci.* **9**, 385 (2015).
36. Vezzani, A., French, J., Bartfai, T. & Baram, T. Z. The role of inflammation in epilepsy. *Nat. Rev. Neurol.* **7**, 31–40 (2011).
37. Kolar, S. L. *et al.* Group B streptococcus evades host immunity by degrading hyaluronan. *Cell Host Microbe* **18**, 694–704 (2015).
38. Luo, S., Ma, K., Wang, Z. F., Lu, C. F. & Liu, Y. J. Prokaryotic expression of hyaluronidase from *Streptococcus agalactiae* and its pathogenicity in mice. *Chin. Vet. Sci.*** **46**, 2645–2655 (2016).
39. Zwijsen, P. J. *et al.* Experimental pneumococcal meningitis in mice: a model of intranasal infection. *J. Infect. Dis.* **183**, 1143–1146 (2001).
40. Liu, G. J., Zhang, W. & Lu, C. P. Comparative genomics analysis of *Streptococcus agalactiae* reveals that isolates from cultured tilapia in China are closely related to the human strain A909. *BMC Genomics* **14**, 775–784 (2013).

**Acknowledgements**

This work was supported by the Natural Science Foundation of Jiangsu Province (BK20171376), Qing Lan Project of Jiangsu Province and the Priority Academic Program Development of Jiangsu Higher Educational Institutions (PAPD).

**Author Contributions**

Y.J.L. and S.L. conceived the study and drafted the paper; S.L., Q.C., K.M., Z.F.W. and G.J.L. performed the experiments; C.P.L. provided valuable suggestions of the manuscript.

**Additional Information**

**Competing Interests:** The authors declare that they have no competing interests.

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2017