

Regulative synthesis of capsular polysaccharides in the pathogenesis of *Streptococcus suis*

Reviewed Preprint

v1 • October 30, 2024

Not revised

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eLife Assessment

This **useful** study uses an intranasal mouse infection model with *Streptococcus suis*, a gram-positive bacterial pathogen that causes severe losses in pigs around the world. The manuscript provides insights that the capsular polysaccharide, one of the virulence factors of this pathogen, contributes to tissue dissemination and neurotropism in the host. However, the evidence is currently **incomplete**, and further experiments and careful interpretation of the current results and methods used are necessary to support the conclusions of the manuscript.

<https://doi.org/10.7554/eLife.101760.1.sa2>

Abstract

Streptococcus suis (*S. suis*) is an important zoonotic pathogen causing substantial economic losses in the swine industry. *S. suis* serotype 2 (SS2) is often isolated from the diseased. *S. suis* expresses capsular polysaccharide (CPS), a virulence factor crucial for their survival in the blood. However, the role of CPS in the pathogenesis of *S. suis* is incomplete. Here, we showed a dynamic regulation of CPS in *S. suis* pathogenesis. In a mouse infection model, an SS2 strain (05ZYH33) was detected in the nasal-associated lymphoid tissue (NALT) and cerebrospinal fluid (CSF) as early as 30 min after intranasal inoculation without bacteremia. Histological analysis revealed that 05ZYH33 in the nasal cavity invaded the olfactory epithelium, resulting in early brain inflammation. Transmission electron microscopy showed that 05ZYH33 isolated from NALT and CSF at early infection time had a thin layer of CPS, and those detected in the blood 5 h post-inoculation showed a much thicker CPS. In addition, adoptive transfer of anti-CPS restricted 05ZYH33 in the blood but not in NALT or CSF. However, an antiserum directed to multiple non-CPS virulence factors (anti-V5) efficiently inhibited 05ZYH33 in NALT, CSF, and blood. Thus, 05ZYH33 colonizes NALT more efficiently without CPS and subsequently invades the meninges through the olfactory nerve system. These findings provide valuable information for the treatment of *S. suis* infection and the development of vaccines across serotypes of *S. suis* by targeting CPS-independent immunity.

Introduction

(A) *S. suis* is a zoonotic pathogen in the porcine industry. Thirty-three serotypes of *S. suis* have been described to date based on the capsular polysaccharides (CPS). The *S. suis* serotype most frequently isolated from diseased swine and humans is serotype 2 (SS2). *S. suis* is commonly found in NALT of asymptomatic piglets. Under some unknown conditions, *S. suis* can reach and survive in the blood and invade the brain to cause meningitis and multi-organ dissemination. Infection of the central nervous system (CNS) is a cause of mortality in piglets with the disease. CPS is essential for survival of *S. suis* in the bloodstream and is recognized as an important virulence factor of this bacterial pathogen. However, the role of CPS in the pathogenesis of *S. suis* and the function of CPS-induced immunity in SS2 protection is controversial [1]. The opsonizing antibodies targeting CPS are protective [2, 3] but only partially protect against infection [4]. Furthermore, a CPS-glycoconjugated vaccine protected parenterally challenged piglets [5], but protection by anti-CPS has not been demonstrated in animals challenged through the intranasal route, which is a common natural infection route of *S. suis*.

Synthesis of CPS is enhanced in the bloodstream and reduced in the CNS [6, 7]. *In vitro* experiments revealed that the presence of CPS attenuated 05ZYH33 invasion of epithelial cells [8–10] and that unencapsulated mutants displayed increased rates of invasion of the blood–cerebrospinal fluid barrier (BCSFB) [8, 9]. These observations suggest that *S. suis* regulates CPS synthesis to fit hosts. However, despite the proposal of this hypothesis more than two decades ago, there is still no direct evidence of such encapsulation modulation for *S. suis* [11].

Two pathways have been exploited by meningeal pathogens to access the CNS: penetrating the blood–brain barrier (BBB) or the BCSFB through the blood or directly accessing the CNS via the olfactory or trigeminal nerves [12]. Some human meningeal pathogens, such as *Streptococcus pneumoniae* and *Neisseria meningitidis*, can directly transmigrate from the nasal mucosa into the CNS through the olfactory nerve [12–14]. Similar to these meningeal pathogens, the predominant route of *S. suis* infection in pigs is through the upper respiratory tract (URT), which may provide the opportunity for *S. suis* to transmigrate directly to the CNS from the nasal cavity.

Studies on *S. suis* commonly use parenteral infection models that bypass colonization of the URT, thus this critical step of *S. suis* pathogenesis is largely unexplored. In the present study, mice were infected intranasally (i.n.) with 05ZYH33, and the infection process was monitored. We show that 05ZYH33 regulated the synthesis of CPS at different stages of infection and that 05ZYH33 migrated to the CNS from the nasal cavity independent of CPS before it entered the blood. Moreover, we revealed that immunity against non-CPS virulence factors of *S. suis* efficiently prevented nasal–CNS transmigration and subsequent progress of the infection.

Results

S. suis serotypes regulated CPS synthesis in different culture phases

A clinical isolate of *S. suis* serotype 2 wild type (05ZYH33) was cultured and collected in the lag, logarithmic (log), and stationary phases [optical density at 600 nm (OD₆₀₀) of 0.20, 0.60, and 0.80, respectively] (Fig. 1A). The CPS of 05ZYH33 collected in different growth phases was quantitated based on hydrophobicity in a bacterial adhesion to hydrocarbon (BATH) assay. High surface hydrophobicity leads to high bacterial adherence to hydrocarbon (high BATH value) and reflects low levels of CPS on the surface of 05ZYH33. As shown in Fig. 1B, the CPS-deficient strain

$\Delta 2BSS2$ (05ZYH33 isogenic), used as a control, exhibited the highest BATH value ($69.5 \pm 6.51\%$). A similarly high BATH value ($62.5 \pm 9.73\%$) was found in the log phase of 05ZYH33. However, the BATH values in the lag and stationary phases of 05ZYH33 were substantially lower ($37.0 \pm 17\%$ and $15.6 \pm 9.2\%$, respectively). These data indicate that CPS of 05ZYH33 was synthesized dynamically *in vitro*, with the highest quantity in the stationary phase and the lowest in the log phase.

To determine the dynamic CPS synthesis in *S. suis* serotypes, four other serotypes of *S. suis* were cultured and collected in the log and stationary phases for CPS quantitation by BATH assays. As shown in **Fig. 1C**, all the serotype strains had high hydrophobicity at the log phase and low at the stationary phase. The similarity in CPS patterns of these strains suggests a shared regulation mechanism by *S. suis* serotypes.

Monitoring the CPS content of *S. suis* serotypes in culture

(A) The growth curve of 05ZYH33 in TSB-FBS culture was assessed by measuring the optical density at 600 nm (OD₆₀₀) at specified time points. Data present the mean $\pm$ SD of three independent experiments ($n = 10$). (B) The hydrophobicity of 05ZYH33 during the lag, log, and stationary (Sta) phases was measured by BATH assays ($n = 4$ to 7). (C) The hydrophobicity of indicated serotype strains in the log and stationary phases was measured by BATH assays. A representative photo of the BATH assay (upper panel) and BATH values (lower panel) ($n = 3$ to 6). $\Delta 2BSS2$ refers to an isogenic strain lacking CPS; NC represents a 05ZYH33 suspension without hydrocarbon. **, $P < 0.01$. ***, $P < 0.001$. ****, $P < 0.0001$.

05ZYH33 in different cultural stages exhibited altered abilities for host cell adherence and resistance to phagocytic killing

05ZYH33 and $\Delta 2BSS2$ were cultured and collected at the log and stationary phases to determine the effects of CPS on the binding of 05ZYH33 to epithelial cells. The adherence assay showed that while $16.5 \pm 5.2\%$ of $\Delta 2BSS2$ was associated with epithelial cells (HEp-2), only $4.96 \pm 3.26\%$ of 05ZYH33 in the log phase and $2.05 \pm 1.58\%$ of 05ZYH33 in the stationary phase were associated with the cells (**Fig. 2A**). The bacterial binding was $\Delta 2BSS2 > 05ZYH33$ in the log phase $> 05ZYH33$ in the stationary phase. Similar binding patterns of 05ZYH33 and $\Delta 2BSS2$ were also observed in the assay using human brain microvascular endothelial cells (hBMECs), the major component of the BBB (**Fig. 2B**). These results indicate that CPS reduces adherence of the bacteria to host cells.

CPS of *S. suis* plays a critical role in the resistance to phagocytosis. 05ZYH33 in different growth phases were co-incubated with the blood from naïve mice to determine the effect of CPS on phagocytic killing. After incubation for 3 h, surviving bacteria in the blood examined by colony-forming units (CFUs) showed that substantially more 05ZYH33 in the log and stationary phases were detected compared with $\Delta 2BSS2$ in the matching growth phases. In addition, there were more surviving 05ZYH33 in the stationary phase compared with that in the log phase (**Fig. 2C**). These results suggested that a lack of CPS may facilitate adherence in a host, while high levels of CPS confer resistance to phagocytic killing in the blood.

The course of 05ZYH33 infection in mice following intranasal inoculation

The dynamic synthesis of CPS associated with differential adherence to host cells and resistance to phagocytosis *in vitro* suggested that 05ZYH33 regulates CPS synthesis for pathogenesis and survival in a host. To determine the role of CPS dynamic synthesis in 05ZYH33 pathogenesis, mice were i.n. inoculated with 05ZYH33 to simulate *S. suis* natural infection in pigs and CFUs in various bodily compartments were assessed at different points over 120 h. By 0.5 h after inoculation, CFUs of 05ZYH33 in the nasal-associated lymphoid tissue (NALT), a pig tonsil homologue and the major niche for *S. suis* in pigs, and the lungs were similar (5×10^5) and gradually reduced with time (**Figs.**

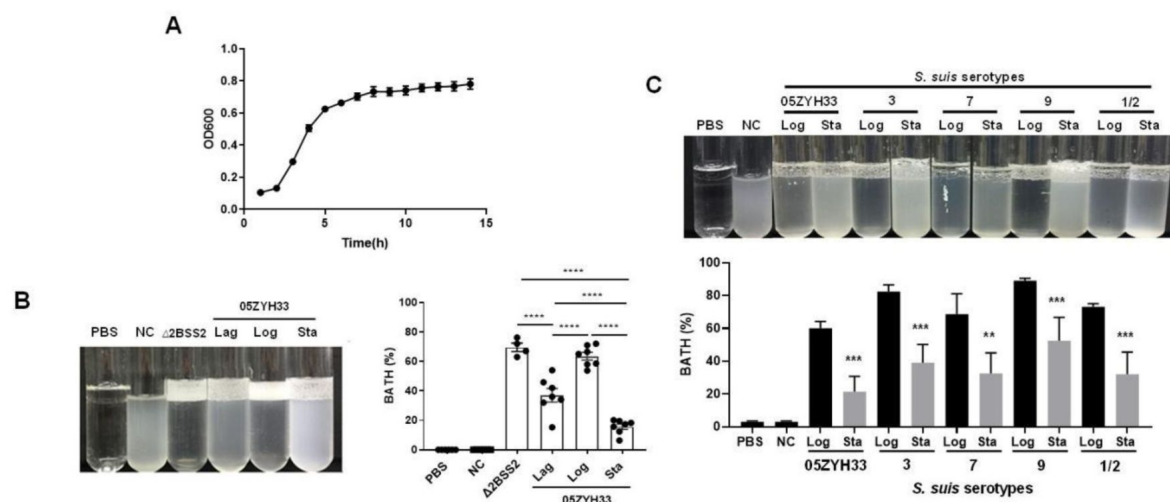


Fig. 1.

Detection of CPS content of *S. suis* serotypes in culture.

(A) The growth curve of 05ZYH33 in TSB-FBS culture was monitored through the optical density at 600 nm (OD₆₀₀) of the culture at the indicated time points. Data are the mean $\pm$ SD of three independent experiments ($n = 10$). (B) The hydrophobicity of 05ZYH33 in the lag, log, and stationary (Sta) phases was measured by BATH assays. A representative photo of the BATH assay (upper panel) and BATH values (right panel). Δ 2BSS2, the isogenic strain of 05ZYH33 without CPS; NC, 05ZYH33 suspension without hydrocarbon ($n = 4$ to 7).

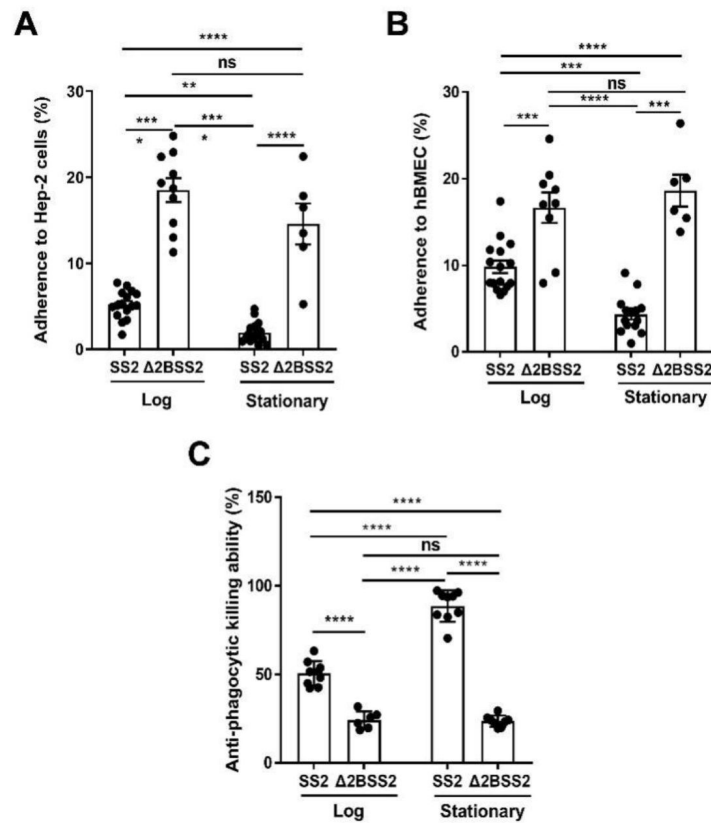


Fig. 2.

The adherence of 05ZYH33 to epithelial cells and its resistance to phagocytosis

HEp-2 or HBMECs cells were inoculated with 05ZYH33 or Δ2BSS2 in either the log or stationary phase at an infection MOI of 10. (A) Adherence rate of *S. suis* to HEp-2 cells. (B) Adherence rate of *S. suis* to HBMECs. (C) *S. suis* strains in log and stationary phases were incubated with whole blood from naïve mice for 3 h, and the viability of *S. suis* was determined. Data are from three independent experiments and present as means ± SEM ($n = 6$ to 16). $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$; ns, not significant.

3A and **3B**), suggesting that the CFUs in the lungs are from the nasal cavity. Up to 10^5 CFUs were also found in CSF samples by 0.5 h (**Fig. 3C**). The curves of CFUs in NALT, the lungs, and CSF showed a similar trend, which was a high level for a few hours followed by a gradual reduction with increasing days. In contrast, 05ZYH33 was not detected in the blood until 5.0 h after inoculation (**Fig. 3D**). As expected, CFUs in the spleen and heart at indicated times (**Figs. 3E** and **3F**) were similar to those in the blood, indicating that they disseminated from the circulation. These findings suggested that 05ZYH33 in the nasal cavity can transmigrate to the lungs and CNS before bacteremia and disseminate to other organs after entering the blood. During the five days (120 h) following inoculation, the mice did not show significant disease symptoms, and their physical conditions were similar compared to non-infected mice (body weight, appetite, and activity). No obvious disease symptoms might be due to the bacteria cleared relatively quickly.

The CPS of 05ZYH33 was synthesized differentially in stages of infection in mice

05ZYH33 was isolated from the bodily compartments of mice 12 h after inoculation. The 12 h of infection allows the detection of the bacteria in different body compartments of the infected mice. Following enriched by culturing the isolates in TSB to an OD_{600} of 0.7 CPS synthesis *in vivo* was determined by BATH assay. The hydrophobicity of isolates from NALT and the CSF was lower compared with that of $\Delta 2BSS2$, but higher than that of the isolates from the blood (**Fig. 4A**). This indicates that 05ZYH33 downregulates CPS synthesis when it colonizes the nasopharyngeal mucosa or enters the CNS and upregulates CPS synthesis upon entering the blood. Transmission electron microscopy (TEM) was performed directly on 05ZYH33 isolated from mice without *in vitro* culture to confirm the BATH assay results. For cultured strains without mouse infection (**Fig. 4Ba**, **b**, and **c**), similar to $\Delta 2BSS2$ (**Fig. 4Ba**), there was no CPS observed on one side of dividing 05ZYH33 cell and only a thin and partial layer of CPS on the other side of the cell in the log phase (**Fig. 4Bb**) and a thicker layer of CPS was observed in the stationary phase (**Fig. 4Bc**). For 05ZYH33 isolated directly from infected mice (**Fig. 4Bd**, **e**, and **f**), the CPS was much thicker in the blood isolates (**Fig. 4Bf**) compared with that of the NALT isolates where it was thinner and much sparser (**Fig. 4Bd**). In contrast, the outermost layer of the isolates from the CSF was highly dense without an apparent CPS layer (**Fig. 4Be**), suggesting that CPS is downregulated, and the membrane components of the CSF isolates are changed. These findings support that CPS is downregulated for colonization and upregulated in the blood to avoid phagocytic killing.

An antiserum against non-CPS virulence factors of *S. suis* reduced 05ZYH33 colonization in NALT, CSF, and lungs

We have previously demonstrated that immunization with multiple non-CPS virulence factors of *S. suis* (named V5) prevents 05ZYH33 colonization of the nasal mucosa and neuropathological symptoms in mice [15]. We hypothesized that the serum against V5 would inhibit the nasal–CNS transmigration of 05ZYH33. Mice were i.n. infused with anti-V5 or anti-CPS serum, inoculated i.n. with 05ZYH33 6 h later, then euthanized 12 h post-inoculation (**Fig. 5A**) to detect the bacteria in different body compartments of the infected mice. In mice that received antisera through the i.n. route, CFUs reduced in all examined compartments of anti-V5 recipients but only reduced in the lungs and blood of anti-CPS recipients (**Fig. 5B**). These results indicated that 05ZYH33 survival in the blood depends on CPS and nasopharyngeal colonization and nasal–CNS transmigration are more dependent on non-CPS molecules of 05ZYH33. In addition, when mice received either anti-V5 or anti-CPS through the intravenous (i.v.) route, CFUs were reduced in the lungs and blood but not in NALT and the CSF (**Fig. 5C**), compared with mice received naïve serum through the i.v. route. These results indicate that systemic immunity can efficiently protect against infection in the lungs and blood but not against colonization in the nasal mucosal and CNS, at least at the early infection time.

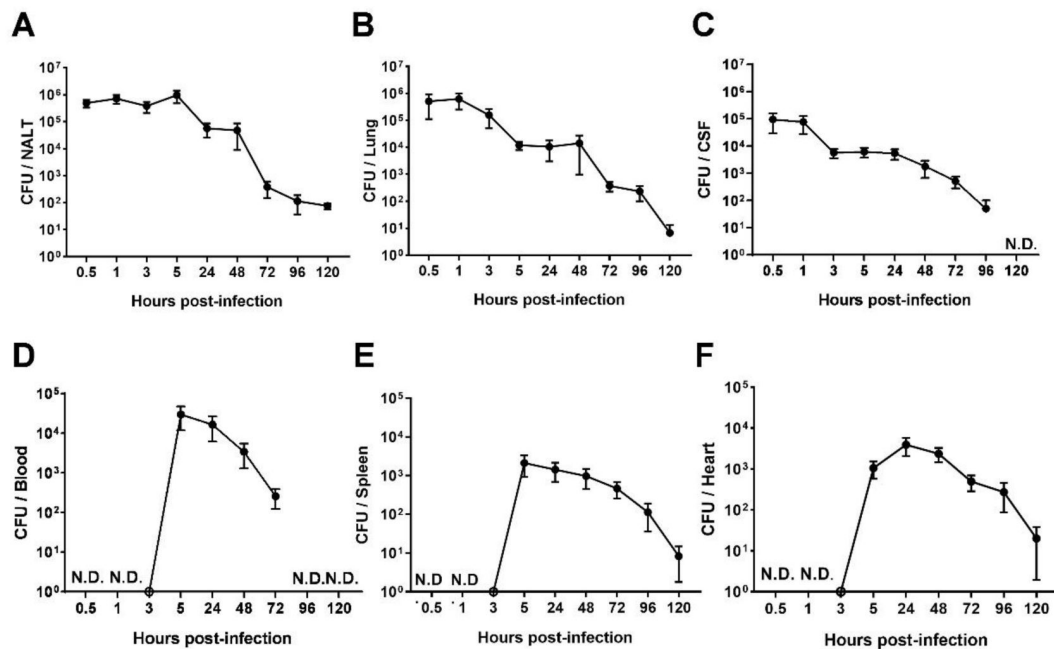


Fig. 3.

The intranasal infection course in mice.

Mice were intranasally inoculated with 05ZYH33. Total CFUs in various bodily compartments were assessed at the indicated time points after inoculation, (A) NALT, (B) the CSF, (C) the lung, (D) the blood, (E) the spleen, and (F) the heart. The data presented are derived from two independent experiments and presented as means $\pm$ SEM ($n = 6$). N.D., not detected.

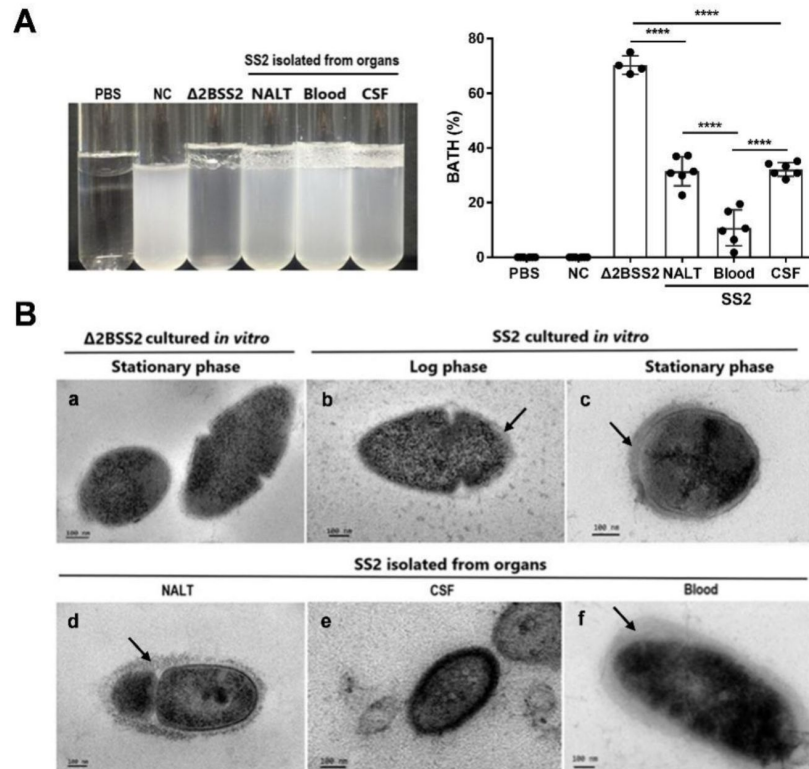


Fig. 4.

The CPS of 05ZYH33 isolated from various bodily compartment following infection.

05ZYH33 was intranasally inoculated in mice. Twelve hour after inoculation, CFUs in NALT, the CSF, and the blood were assessed for (A) hydrophobicity of bacterial cells by BATH assay and (B) CPS by TEM. Arrows indicate the CPS layers. Scale bar, 100 nm. Data in (A) are from two independent experiments and present as means $\pm$ SEM ($n = 4$ to 6). NC, 05ZYH33 suspension without hydrocarbon. **** $P < 0.0001$.

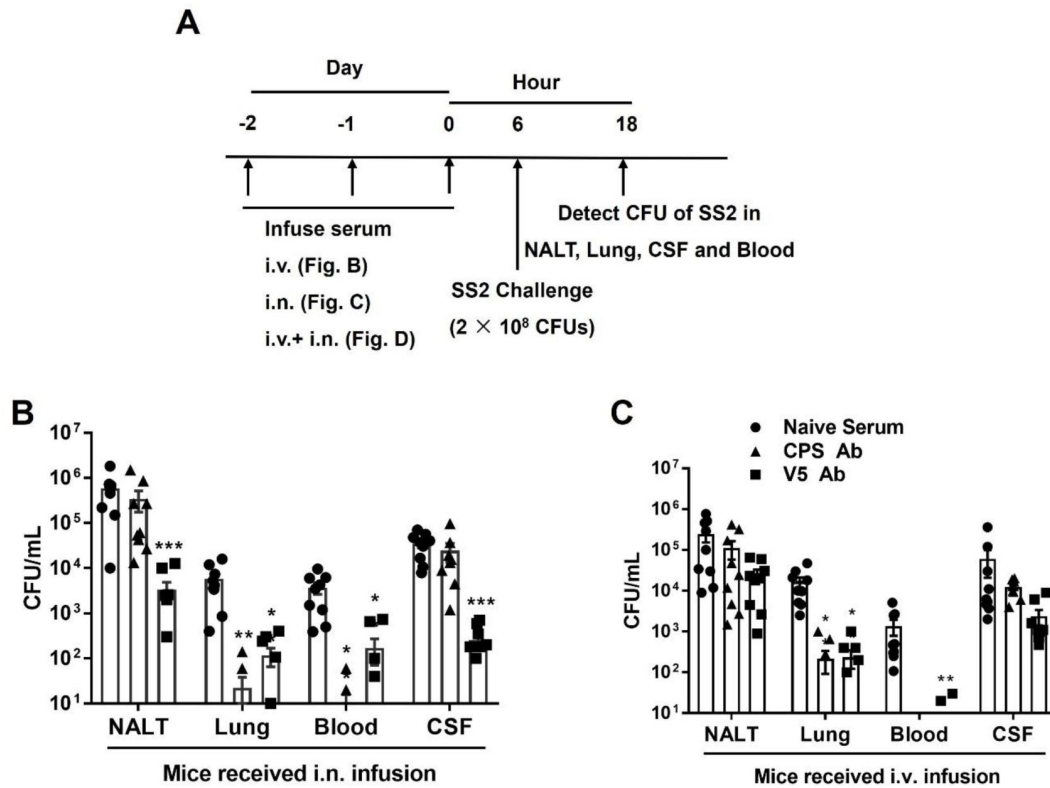


Fig. 5.

The role of anti-CPS or anti-V5 serum in 05ZYH33 infection.

(A) Schematic illustration of passive transfer of antisera and infection in mice. (B) and (C) Anti-V5 or anti-CPS antiserum was transferred through the i.n. or i.v. route. Six hours later, mice were i.n. challenged with 05ZYH33, then euthanized 12 h post-challenge. CFUs of 05ZYH33 in NALT, the lungs, the blood, and the CSF were determined. Data are the mean $\pm$ SEM of three independent experiments ($n = 9$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Early 05ZYH33 colonization in the CNS was reduced by anti-V5 but not anti-CPS serum

The simultaneous early 05ZYH33 colonization in NALT and CNS support a nasal–CSF transmigration. To exclude the possibility that CFUs in the CNS at 12 h post-infection is disseminated from the circulation, mice received antisera via the i.n. route and were inoculated with 05ZYH33 as before, then CFUs were determined 1 h post-inoculation before the bacteria entered the blood (**Fig. 6A**). As shown in **Fig. 6B**, CFUs in NALT and the CSF of anti-CPS recipients were similar to those in naïve-serum recipients; however, those in NALT and the CSF of anti-V5 recipients were 10-fold lower compared with those of the naïve-serum recipients. To confirm that the nasal–CNS transmigration is independent of CPS, mice received naïve serum or anti-V5 via the i.n. route, then were inoculated with $\Delta 2BSS2$. One hour later, we found 4.5×10^5 CFUs in NALT and 2.1×10^5 CFUs in the CSF of naïve-serum recipients. However, CFUs were 10-fold and 100-fold lower in NALT and the CSF, respectively, of anti-V5 recipients compared with those in naïve-serum recipients (**Fig. 6C**). These results demonstrate that 05ZYH33 in the CNS in the early infection is from the nasal cavity independent of CPS.

The olfactory nerve is the invasion route of 05ZYH33 for nasal–CNS transmigration

The olfactory nerve is a port of entry to the meninges for some human meningeal pathogens and is the likely the route of the early CNS dissemination by 05ZYH33. Histological analysis of the anatomical structures in the distal nasal cavity and olfactory epithelium in **Fig. 7A** showed that 1 h post-infection, the bacteria were closely associated with ciliated respiratory epithelium in the nasal cavity (NC) and grouped bacteria were spread in the subepithelial tissue with significant tissue damage (**Fig. 7A, b**). Scattered bacteria were in close contact with the olfactory epithelium (OE) and lamina propria (LP), accompanied by severely destroyed tissue (indicated by the loss of immunofluorescence signal in the area) (**Fig. 7A, d**). Large groups of bacteria were observed within the olfactory bulb (OB) (**Fig. 7A, f**). These observations suggest that 05ZYH33 in the nasal cavity could invade the olfactory epithelium through the sensory neurons protruding into the NC and subsequently enter the brain along the sensory neurons at the cribriform plate. Histological analysis of the brain in 05ZYH33-infected mice revealed that inflammatory cells were infiltrated in the brain tissue with surrounding edema at 1 h post-inoculation (**Fig. 7B, c** and **d**). The pathological changes were more severe 9 days after 05ZYH33 inoculation (**Fig. 7B, e** and **f**), which could be resulted from both nasal–CNS transmigration and 05ZYH33 dissemination from the blood. Thus, nasal–CNS transmigration is an efficient route of *S. suis*-mediated CNS infection.

Mice were pretreated with acetic acid to increase the sensitivity to 05ZYH33 infection [15]. To exclude that nasal–CNS transmigration results from irritation of the nasopharyngeal mucosa by acetic acid, mice were infected without acid treatment (pretreated with PBS). One hour post-inoculation CFUs were found in the CSF of the mice even though the numbers were lower than those of acidic acid-treated mice (**Fig. 7C**). CFUs were also detected in the CSF when mice were infected with $\Delta 2BSS2$ without acid treatment (**Fig. 7D**). These results indicated that nasal–CSF transmission takes place without acidic acid and independent of CPS and that irritation of the nasopharyngeal mucosa facilitates nasal–CNS transmission.

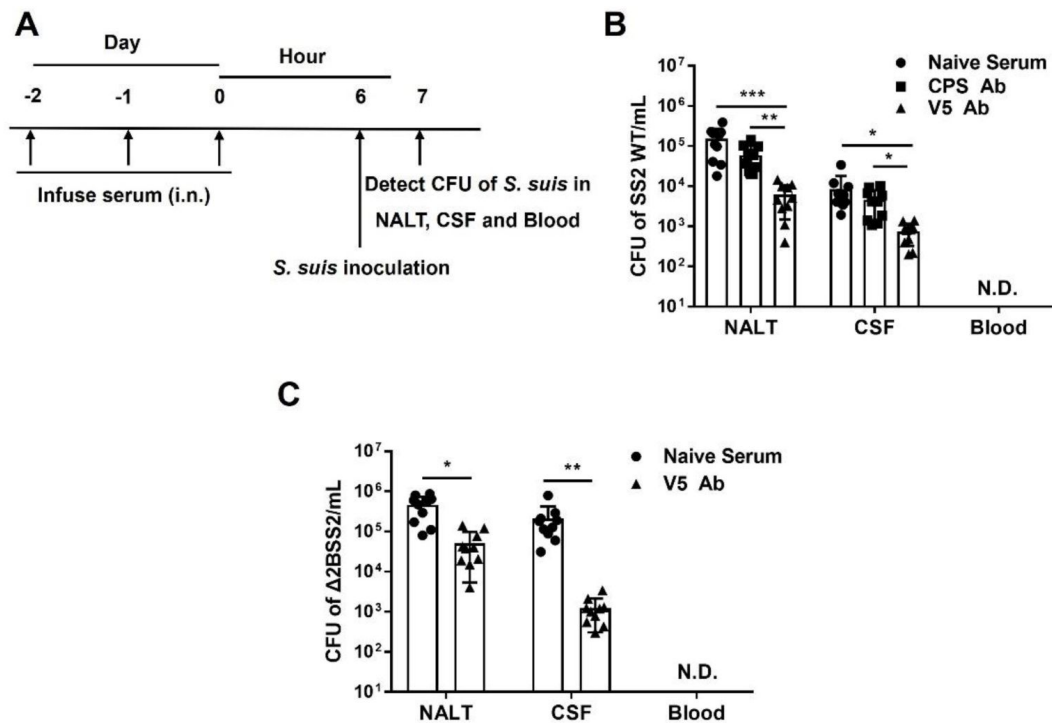


Fig. 6.

Detection of 05ZYH33 colonization in the CSF at early time of infection.

(A) Schematic illustration of passive transfer and mouse infection. (B) and (C) Mice were infused with anti-V5 serum through the i.n. route, and inoculated i.n. with 05ZYH33 or Δ2BSS2 6 h later. One hour later, mice were euthanized, and CFUs in NALT, the blood, and the CSF were determined. Data are the mean ± SEM of three independent experiments ($n = 10$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

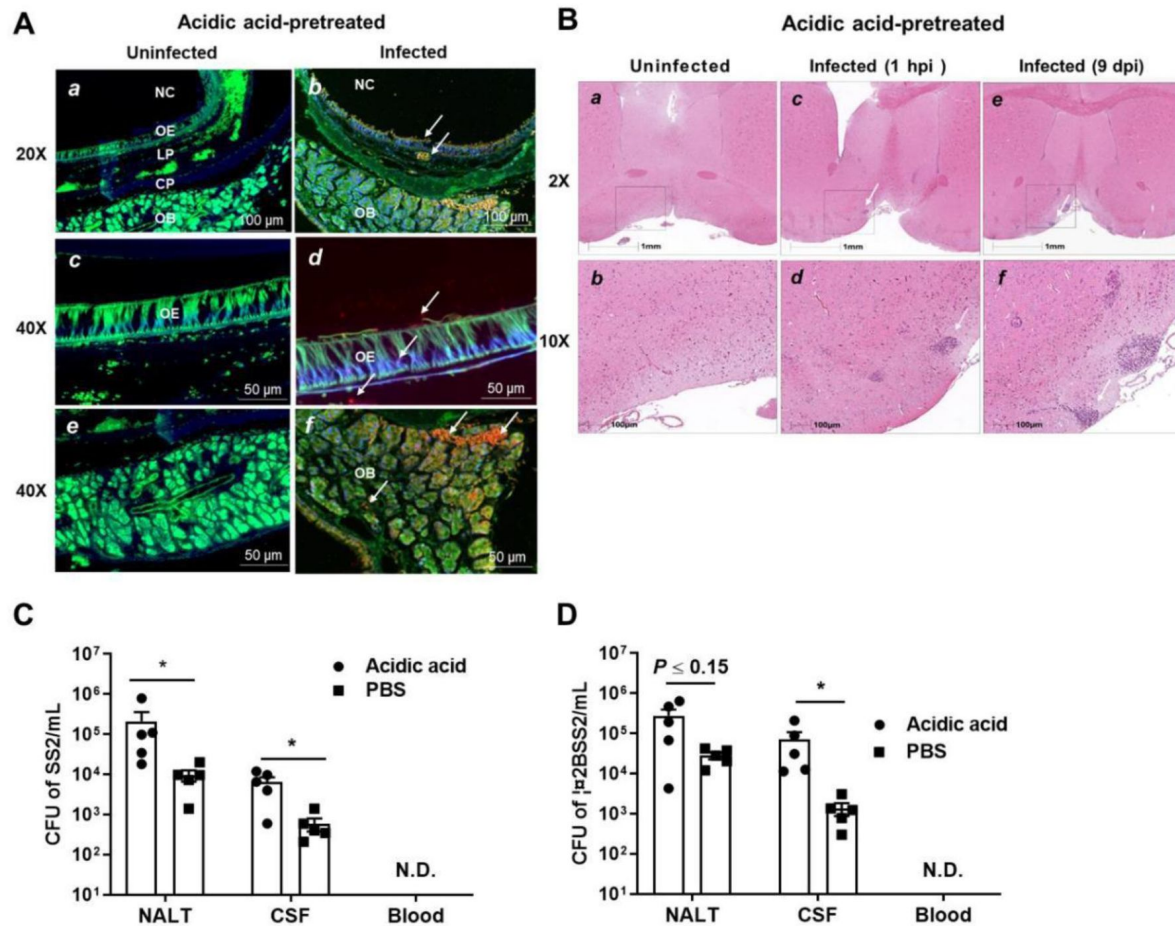


Fig. 7.

Detection of the presence of 05ZYH33 in the tissue of olfactory nerve area.

Mice were inoculated with acetic acid through the nostril and one hour later infected i.n. with 05ZYH33. (A) Sagittal views of the olfactory system. Sections of the distal nasal cavity and olfactory epithelium from uninfected (a, c, and e) and 05ZYH33-infected (b, d, and f) mice. Red or orange, 05ZYH33; green, the neuronal marker β -tubulin III; blue, DNA. NC, Nasal cavity; OE, olfactory epithelium; LP, lamina propria; CP, cribriform plate; OB, olfactory bulb. (B) One hour or nine days after 05ZYH33 infection, the brain of mice were sectioned for H&E staining. Arrows indicate infiltrated inflammatory cells. (C) and (D) Mice were inoculated with acetic acid or PBS and one hour later infected i.n. with 05ZYH33 or Δ 2BSS2. (C) CFUs of 05ZYH33 or (D) Δ 2BSS2 in NALT, the CSF, and blood were determined one h after infection.

Discussion

CPS is an important virulence factor of *S. suis*, but its role in *S. suis* pathogenesis is controversial. Most animal models in studies of *S. suis* employ the intraperitoneal (i.p.) or i.v. route for infection, which bypass the nasopharynx, the natural infection route of *S. suis*. In this study, CPS thickness of *S. suis* SS2 strain 05ZYH33 was monitored in an i.n. infection mouse model. The results indicate that 05ZYH33 downregulates CPS synthesis to promote nasopharyngeal colonization and nasal–CNS transmigration at early time and upregulates CPS to escape phagocytic killing later in the blood. The study also indicates that CPS-independent immunity can prevent nasal–CNS transmigration and the progression of 05ZYH33 infection.

Clinical CNS symptoms in pigs are often preceded by a phase of bacteremia [16, 17], indicating that *S. suis* meningitis results from the dissemination of bacteria from the blood through the BBB or the blood–CSF barrier. Our study revealed that, like some human meningeal pathogens, the olfactory nerve epithelium provides an alternative route for 05ZYH33 invasion to the CNS. Swine influenza virus (SIV) infection was reported to promote *S. suis* invasion of deeper tissues by damaging epithelial cells [18–20]. Nasopharyngeal inflammation by co-infected *S. suis* and SIV could cause tissue damage to the olfactory epithelium, which may exacerbate nasal–CNS entry. As the mouse is not a natural host of *S. suis* and is insensitive to the infection, a relatively high dosage is needed for optimal bacterial retention in NALT, which can lead bacteria into the lungs. Thus, early 05ZYH33 colonization in the lungs of this model may not reflect the state of *S. suis* infections in pigs. The early 05ZYH33 colonization in NALT and the CSF with similar dynamics suggests that meningitis could be early manifestations of *S. suis* disease before multi-organ dissemination through the blood. The findings in the study indicate that restriction of *S. suis* proliferation in NALT is critical to prevent the severity and lethality of *S. suis* disease.

Although CPS plays an important role in *S. suis* survival in the blood, it is not the only component required for the event [21, 22]. Similar to that observed with anti-CPS, infusion of anti-V5 through the i.v. route reduced 05ZYH33 in the blood and lungs, indicating that blocking CPS-independent mechanisms can restrain the progression of 05ZYH33 infection and suggests that non-CPS virulence factors, such as those in V5, are required for 05ZYH33 survival in the blood and lungs. Furthermore, although anti-CPS promoted 05ZYH33 clearance in the blood, it did not prevent 05ZYH33 colonization of the nasal cavity and invasion of the CNS. However, anti-V5 reduced 05ZYH33 in all examined compartments when administered via the i.n. route, supporting that inhibition of nasal colonization by CPS-independent immunity is critical to prevent the progression of the disease. Moreover, the nasal–CNS route may not be the main pathway for clinical meningitis compared to the blood–CNS route. However, mucosal blockage of nasal colonization reduces CFU in the CSF more effectively than systemic antibody, indicating reduction of nasal colonization at early time would significantly prevent the bacteria from entering the CNS through the early nasal and later blood route.

In summary, this study supports an updated *S. suis* infection model. Briefly, 05ZYH33 downregulates CPS to colonize the URT mucosa, where it can transmigrate to the CNS through the olfactory epithelium early in the infection process. 05ZYH33 upregulates CPS to avoid phagocytic killing when it enters the blood, leading to multi-organ dissemination. The regulation of CPS at different stages of 05ZYH33 pathogenesis and the protection offered by CPS-independent immunity provide valuable information for clinical treatment and development of vaccines across serotypes of *S. suis*.

Materials and methods

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the IMCAS (Institute of Microbiology, Chinese Academy of Sciences) Ethics Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of IMCAS (permit no. APIMCAS2019004). Mice were bred under specific pathogen-free (SPF) conditions in the laboratory animal facility at IMCAS. All animal experiments were conducted under isoflurane anesthesia, and all efforts were made to minimize suffering.

Bacterial strains and growth conditions

S. suis strain SS2 05ZYH33 (GenBank no. for complete genome sequence: CP000407.1) was isolated from a fatal human case of streptococcal toxic shock-like syndrome (STSLs) in 2005 in Sichuan province, China. A capsular mutant of *S. suis* 05ZYH33 (Δ 2BSS2) was obtained from Prof. Jia-Qi Tang (General Hospital of the Eastern Theater of the People's Liberation Army, China) [23]. *S. suis* serotypes SS3, SS7, SS9, and SS1/2 were gifts from Dr. Shengbo Cao (National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China). The information of these strains is listed in Supplementary Table 1. All *S. suis* strains were cultured in tryptic soy broth (TSB) containing 5% newborn bovine serum (FBS) or on tryptic soy agar (TSA) supplemented with 5% FBS at 37°C and 5% CO₂. The cultured strains were washed and resuspended in phosphate-buffered saline (PBS). CFUs were verified by plating on blood agar.

Growth curve of 05ZYH33

05ZYH33 was grown in TSB-FBS medium to an OD₆₀₀ of approximately 0.4 and then diluted 1000 times with fresh medium. Next, 200 μ L of the bacterial suspension was added in quadruplicate to a 100-well honeycomb plate and cultured at 37°C and 5% CO₂ for 16 h. The OD₆₀₀ of each well was measured at different times using a CMax Plus plate reader (Molecular Devices, San Jose, CA, USA).

Determination of CFUs in various bodily compartments

Mice were infected i.n. with 05ZYH33 (stationary phase). Then at different time points, the lung, spleen, heart, and NALT were taken from mice, and the blood and CSF were collected. A single-cell suspension of each organ was prepared in 1-5 mL PBS. CFUs in the single cell suspension, the blood, and the CSF were determined by plating serially diluted samples on blood agar plates and expressed as total CFUs in each organ or in the blood and CFS by following formula: CFUs on the plate $\times$ dilution factor $\times$ total volume of a sample.

Bacterial adhesion to hydrocarbon (BATH) assay

The hydrophobicity of *S. suis* was assessed by a modified BATH assay [24]. Briefly, 05ZYH33 cells in cultures were collected, washed, and resuspended in PBS, adjusting the OD₆₀₀ of the suspensions to 2.0 ($\pm$ 0.2) with PBS. Four milliliters of each suspension were added to a tube containing 300 μ L xylene and 300 μ L hexadecane. A negative control comprised a 4 mL bacterial suspension without hydrocarbon. After vortexing for 20 s, the tubes were maintained at 37°C for 30 min. The OD₆₀₀ of the resulting lower aqueous layer and the tube contents before vortexing were measured. The ratio of the absorbance of the bacteria assay tubes (Ab) to the absorbance of the bacteria control (Ac) was calculated as adhesion to hydrocarbon (%) = (Ac - Ab) / Ac $\times$ 100.

Bacterial adherence assay

The human laryngeal epithelial cells (HEp-2, CL-0104) were purchased from Procell Life Science & Technology Co., Ltd. Wuhan, Hubei Province and cultured according to the manufacturer's instruction. The human brain microvascular endothelial cells (hBMEC) were kindly provided by Prof. Xiang-Ru Wang in Huazhong Agricultural University, Wuhan, Hubei, China and cultured as described by Yang [25]. Adherence of 05ZYH33 or Δ2BSS2 in log and stationary phases was performed as described previously with slight modifications [26]. Cells were cultured in 24-well plates to 80–90% confluence ($\sim 1.2 \times 10^5$ cells/well), then starved overnight in DMEM without serum and antibiotics. Cell monolayers were washed with PBS and overlaid with DMEM medium with 10% FBS (500 μ L/well) containing 05ZYH33 or Δ2BSS2 (MOI = 10). After incubation for 2 h at 37°C and 5% CO₂, unbound bacteria were removed, and the cells were lysed with 0.025% Triton X-100. CFUs in samples of diluted cell lysate were determined on blood agar plates and expressed as the percentage of adherence: (CFUs in wells with cells / CFUs in wells without cells) $\times$ 100.

Anti-killing assay

Phagocytosis assays were performed as described by Pian et al. [27] with slight modifications. Briefly, 1×10^5 05ZYH33 or Δ2BSS2 in log and stationary phases were added to whole blood from naïve mice. The mixtures were rotated for 3 h at 37°C and 5% CO₂. A set of the same bacterial samples was added to naïve serum to determine bacterial numbers in the 3 h incubation without killing. Viable bacterial counts were determined by plating diluted samples on blood agar. The anti-killing capacity was calculated as (CFU in blood / CFU in serum) $\times$ 100%.

Mouse infection

C57BL/6J Cnc wild-type mice (aged 6 to 7 weeks) were purchased from Vital River Laboratory Animal Center (Beijing, China) and randomly assigned to groups. Mice were pretreated with 5 μ L of 1% acetic acid (pH 4.0) or PBS per nostril 1 h before intranasal infection [15]. After further anesthesia, mice were inoculated intranasally with 05ZYH33 (stationary phase, 2×10^8 CFU / 10 μ L). 5 μ L of the inoculum was delivered intranasally to each nostril (total 10 μ L). This volume allows optional bacterial retention in NALT.

Specimen collection and preparation

Mice were euthanized, then blood samples were collected from the heart, and the lungs, heart, spleen, and NALT were removed and washed with PBS to eradicate blood contamination. Single-cell suspensions of the tissues were prepared by mechanical dissociation of tissue through a 100- μ m nylon mesh in 1 mL PBS. CSF samples were obtained by soaking brain tissue in 1 mL PBS for 1 h after sufficient laceration. Supernatants of the single-cell suspension and the blood and CSF samples were used to detect CFU and examine the CPS.

Transmission electron microscopy (TEM)

S. suis cells were enriched by gradient centrifugation of homogenized NALT and samples of the blood and CSF of infected mice. After washing, the bacteria were fixed in 2% paraformaldehyde supplemented with 0.05% ruthenium red in 0.1 M phosphate buffer (pH 7.0) for 30 min at room temperature. The bacterial samples were further prepared and embedded in epoxy resin (Epon 812, Merck) as described by Bojarska et al. [28]. Ultrathin (70–80 nm) sections were cut, stained with 1.2 % uranyl acetate and 2.5 % lead citrate, and then examined with a JEM-1400 (120 kV) transmission electron microscope (JEOL, Tokyo, Japan). Images were taken with a Morada digital camera (Olympus SIS, Münster, Germany) at 10 M pix resolution.

Cloning and expression of recombinant V5 proteins

V5 contains five conserved virulence factors of *S. suis*. They are SrtA (amino acids 50 to 249), ScpCL (amino acids 36 to 535), MRP (amino acids 283 to 721), SLY (amino acids 28 to 497), and SspA (amino acids 41 to 852) and prepared as described by Xing X. et al. (15). Briefly, the genes of V5 were cloned from the genome of *S. suis* strain A7 (serotype 2) (GenBank accession number: NC_017622.1). The recombinant proteins of V5 were expressed in *E. coli* BL21 (DE3) and purified by immobilized metal ion affinity chromatography (IMAC) to reach 95% purity. Bacterial lipopolysaccharide was removed following purification (≤ 0.1 EU/ μ g). Protein concentrations were determined using the BCA Protein Assay Kit according to the manufacturer's instructions (TIANGEN, catalog number: PA115-02).

Preparation and purification of anti-CPS and anti-V5 antibodies

A female New Zealand white rabbit (SPF, 2.0–2.5 kg) was purchased from JinMuYang Laboratory Animal Center (Beijing, China) and kept in the SPF animal facility for 1 week. V5 (1 mg) was mixed with 1 mL incomplete Freund's adjuvant (Becton Dickinson, Franklin Lakes, NJ, USA) and subcutaneously injected into the rabbit 4 times at 1-week intervals. Blood samples were harvested 10 days after the last immunization, and the serum was isolated. Anti-CPS (anti-*S. suis* serotype 2) serum was purchased from SSI Diagnostica (Hillerød, Denmark) and purified by absorption with V5 by Ni-NTA agarose (Qiagen, catalog no. 30210) according to the manufacturer's instructions. Briefly, 200 μ L of V5 (His-tagged, 1 μ g/ μ L) were loaded on the 5 mL Ni-NTA agarose column. The collected antiserum was further purified by incubation with formalin-inactivated Δ 2BSS2 (5×10^{10} CFU) overnight at 4°C. The purified anti-CPS recognized 05ZYH33 but not Δ 2BSS2 as determined by enzyme-linked immunosorbent assay (ELISA) against 05ZYH33 and Δ 2BSS2, respectively. Titers of the purified antisera against V5 and CPS were evaluated by ELISA and stored at -80°C until use.

Passive antiserum transfer

Female C57BL/6J Cnc mice (6–8 weeks) were transferred with anti-CPS or anti-V5 serum through the i.n. or i.v. route. Optimal amounts of antisera were predetermined in mouse infection assays (the minimum amount of anti-V5 to inhibit 05ZYH33 colonization in NALT and the minimum amount of anti-CPS to prevent bacteremia). For anti-CPS serum, 20 μ g/200 μ L (100 μ g/mL) was used for tail vein injection or 5 μ g/30 μ L (170 μ g/mL) for nasal drip. For anti-V5 serum, 100 μ g/200 μ L (500 μ g/mL) and 50 μ g/30 μ L (170 μ g/mL) were used through the i.v. and i.n. routes, respectively. Six hours after antiserum transfer, mice were inoculated via the i.n. route with 2×10^8 CFU of 05ZYH33, and CFUs in different compartments of the infected mice were determined.

Immunofluorescence staining and confocal microscopy

Immunofluorescence staining and confocal microscopy of the nasal cavity were performed as described by Pagelow et al. [29]. Briefly, whole heads of the mice were fixed with 4% PFA for at least 24 h before embedding in paraffin wax. Sections of longitudinally bisected heads were deparaffinized followed by antigen retrieval. Immunostaining was conducted using a primary rabbit anti-*S. suis* antibody (1:100) and a mouse anti-tubulin beta III antibody (Merck Millipore, Darmstadt, Germany catalog no. mab5564, 1:300) to detect neuronal structures, and then appropriate fluorophore-conjugated secondary antibodies: donkey anti-rabbit Cy3 (Jackson Immuno-Research Laboratories, Ely, United Kingdom, catalog no. 711-166-152, 1:1000), donkey anti-mouse FITC (Jackson ImmunoResearch Laboratories, Ely, United Kingdom, catalog no. 715-096-150, 1:500). Tissues were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA. catalog no. H-1200). Images were captured using a Leica Aperio Versa 200 scanning microscope and color-balanced uniformly across the field of view using the Image Processing Leica Confocal and ImageJ Software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Histological analysis of brain tissue

Mice were treated with 5 μ L of 1% acetic acid (pH 4.0) per nostril. One hour later, one group of mice was inoculated i.n. with 05ZYH33 (2×10^8 CFU). A control group of mice was given PBS via the i.n. route. Infected mice were euthanized 1 h and 9 days after infection, respectively. The brain was removed and quickly fixed with 4% paraformaldehyde. After paraffin embedding, tissue sections were stained with hematoxylin and eosin (H&E) according to a standard protocol and examined using a Leica SCN400 slide scanner (Leica Microsystems, Germany).

Statistical analysis

Statistical analyses were performed in GraphPad Prism software, version 7.0. CFUs were analyzed by two-tailed unpaired Mann–Whitney U nonparametric *t*-tests. An unpaired, two-tailed Student's *t*-test was used to analyze the statistical significance between two groups. One-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to analyze the statistical significance of differences among more than two groups. A *P*-value of <0.05 was considered significant.

Acknowledgements

The authors acknowledge Prof. Jiaqi Tang of General Hospital of the Eastern Theater of the People's Liberation Army of China for providing the capsular mutant of *S. suis* 05ZYH33 (Δ 2BSS2), Dr. Shengbo Cao (National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China) for sharing *S. suis* serotype strains, Prof. Xiang-Ru Wang in Huazhong Agricultural University, Wuhan, Hubei, China for the human brain microvascular endothelial cells (hBMEC), and Dr. Junfeng Hao in Pathological Laboratory of Institute of Biophysics, CAS, for performing the immunofluorescence microscopy. This research was supported by the National Natural Science Foundation of China grant 31872743 (to B.W.) and the China Postdoctoral Science Foundation Grant 2019M650875 (to X. W.).

Author contributions

B.W. conceived the original idea, designed the experiments and wrote the manuscript. X.W. performed the experiments, analyzed the data, and drafted the manuscript. J.W., N.L., and X.F. contributed to mouse experiments and provided advice on experimental designs.

Competing interests

The authors declare no competing interests.

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Reviewer #1 (Public review):

Summary:

The manuscript by Wang et al. investigates the interesting relationship between *Streptococcus suis* (*S. suis*) growth phases and levels of virulence factor, specifically the capsular polysaccharide (CPS), in the bacterial cell wall. *S. suis* is a gram positive bacterial pathogen that causes important losses in the swine industry worldwide. Interestingly, *S. suis* is also a resident bacteria in the pig tonsils. Vaccination against bacterial infections such as *S. suis* can be difficult, and understanding how the serotype of a bacterial pathogen impacts what body sites are infected and the dynamics of pathogen dissemination is critical. In this case, this manuscript looks at neuroinvasion of *S. suis* following intranasal delivery because this pathogen causes meningitis in infected hosts. Further, understanding host - pathogen interactions at early time points in the upper respiratory tract may have broad implications for vaccine development.

The authors use an understudied mouse intranasal infection model of *S. suis* to connect growth phase related CPS abundance to the pathogenicity of the bacteria in the nose and blood.

Adoptive transfer of serum against either CPS or V5 (five other virulence factors) supports the idea that *S. suis* CPS levels are an important factor that shapes how this bacterium reaches

different organs.

Some conclusions are not completely supported by the present data, and at times the manuscript is disjoint and hard to follow. While the work has some interesting observations, additional experiments and controls are warranted to support the claims of the manuscript .

Strengths:

The model of intranasal infection is compelling to expand upon work previously done in vitro and with systemic routes of infection. The histology and fluorescent imaging of the olfactory epithelium and olfactory bulb complement work in figure 2 about the attachment of *S. Suis* to epithelial cells and the bacterial burden over time in different organs of figure 3. Histology was performed at 1 hour and 9 days after intranasal infection with stationary phase *S. Suis* and drives home that this pathogen can invade the olfactory nerve and may potentially cause bacterial meningitis seen in some infected swine.

The adoptive transfer of either anti-CPS or anti-V5 to mice before infection at both longer (12 hr), and shorter (1 hr) time points is useful to demonstrate that the changes in cell wall composition between the NALT/CSF and blood compartments result in different efficacy in clearing bacteria from those locations. This is fundamental for the development of vaccines for the swine industry and begs those developing other bacterial vaccines to consider what virulence factors are the most useful as neutralizing antibody targets at the sight of bacterial invasion.

Demonstrating that the amount of CPS within the cell wall of *S. suis* is related to the growth phase of the bacteria is an important consideration for vaccine development. While others had previously shown that CPS levels were higher in the blood than in the CNS, and that CPS decreases the invasion of epithelial cells, the close look at the olfactory epithelium at an early time point of 1 hr ties together in vitro findings. The control of a CPS-negative strain was critical to understanding their findings. The location and the microbial community that bacterial pathogens live within may change the growth phase and therefore also the cell wall components.

Weaknesses:

While the authors present compelling data that is relevant to the development of anti-bacterial vaccinations, the data does not completely match their assertions and there are places where some further investigation would further the impact of their interesting study.

Major concerns for the manuscript:

-The intranasal infections were done with *S. suis* in the stationary phase which has been shown to have less CPS on the cell wall. While this mimics the literature that shows *S. Suis* to have less CPS in the CNS, the difference in the pathogenesis of a log phase vs. stationary phase intranasal infection would be interesting. Especially because the bacteria is a part of the natural microbial community of swine tonsils, it is curious if the change in growth phase and therefore CPS levels may be a causative reason for pathogenic invasion in some pigs.

-The authors should consider taking the bacteria from NALT/CSF and blood and compare the lag times bacteria from different organs take to enter a log growth phase to show whether the difference in CPS is because *S. suis* in each location is in a different growth phase. If log phase bacteria were intranasally delivered, would it adapt a stationary phase life strategy? How long would that take?

-Authors should be cautious about claims about *S. suis* downregulating CPS in the NALT for increased invasion and upregulating CPS to survive phagocytosis in blood. While it is true that the data shows that there are different levels of CPS in these locations, the regulation and

mechanism of the recorded and observed cell wall difference are not investigated past the correlation to the growth phase.

- The mouse model used in this manuscript is useful but cannot reproduce the nasal environment of the natural pig host. It is not clear if the NALTs of pigs and mice have similar microbial communities and how this may affect the pathogenesis of *S. Suis* in the mouse. Because the authors show a higher infection rate in the mouse with acetic acid, they may want to consider investigating what the mouse NALT microenvironment is naturally doing to exclude more bacterial invasion. Is it simply a host mismatch or is there something about the microbiome or steady-state immune system in the nose of mice that is different from pigs?

-I have some concerns regarding the images shown for neuroinvasion because I think the authors mistake several compartments of the mouse nasal cavity as well as the olfactory bulb. These issues are critical because neuroinvasion is one of the major conclusions of this work.

<https://doi.org/10.7554/eLife.101760.1.sa1>

Reviewer #2 (Public review):

In this manuscript from Wang et al., the authors seek to examine the role of capsular polysaccharides (CPS) in invasive *S. suis* pathogenesis. They show that CPS thickness variations associate with isolation from different compartments within the infected mouse and that CPS promotes resistance to blood borne immune mechanisms. The authors conclude that thick CPS inhibits colonization/invasion of the NALT and rather antisera against non-CPS. These results are interesting and thought provoking and provide the continued basis for future experiments that delve further into immune mechanisms. However, there are serious concerns about data collection and interpretation that require further data to provide an accurate conclusion. Some of these concerns are highlighted below:

In figure 2, the authors conclude that high levels of CPS confer resistance to phagocytic killing in blood exposed *S. suis*. However, it seems equally likely that this is resistance against complement mediated killing. It would be important to compare *S. suis* killing in animals depleted of complement components (C3 and C5-9).

Intranasal administration non-CPS antisera provides a nice contrast to intravenous administration, especially in light of the recently identified "blood-olfactory barrier". Can the authors provide any insight into how long and where this antibody would be located after intranasal administration? Would this be antibody mediated cellular resistance, or something akin to simple antibody "neutralization"

The micrographs in Figure 7 depict anatomy from the respiratory mucosa. While there is no histochemical identification of neurons, the tissues labeled OE are almost certainly not olfactory and in fact respiratory. However, more troubling is that in figures 7A,a,b,e, and f, the lateral nasal organ has been labeled as the olfactory bulb. This undermines the conclusion of CNS invasion, and also draws into question other experiments in which the brain and CSF are measured.

Micrographs of brain tissue in 7B are taken from distal parts of the brain, whereas if olfactory neuroinvasion were occurring, the bacteria would be expected to arrive in the olfactory bulb. It's also difficult to understand how an inflammatory process would be developed to this point in the brain -even if we were looking at the appropriate region of the brain -within an hour of inoculation (is there a control for acetic acid induced brain inflammation?). Some explanations about the speed of the immune responses recorded are warranted.

The detected presence of *S. suis* in the CSF 0.5hr following intranasal inoculation is difficult to understand from an anatomical perspective. This is especially true when the amount of *S. suis* is nearly the same as that found within the NALT. Even motile pathogens would need far longer than 0.5hr to get into the brain, so it's exceedingly difficult to understand how this could occur so extensively in under an hour. The authors are quantifying CSF as anything that comes out of the brain after mincing. Firstly, this should more accurately be referred to as "brain", not CSF. Secondly, is it possible that the lateral nasal organ -which is mistakenly identified as olfactory bulb in figure 7- is being included in the CNS processing? This would explain the equivalent amounts of *S. suis* in NALT and "CSF".

To support their conclusions about neuroinvasion along the olfactory route and /CSF titer the authors should provide more compelling images to support this conclusion: sections stained for neurons and *S. suis*, images of the actual olfactory bulb (neurons, glomerular structure etc).

<https://doi.org/10.7554/eLife.101760.1.sa0>