Streptococcal Meningitis Reveals the Presence of Residual Streptococci and Down-Regulated Aquaporin 4 in the Brain

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Short Report

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Abstract

The pathology of streptococcal meningitis is poorly understood, even though streptococcal infection induces meningitis. The aim of this study was to clarify the relationship between streptococcal meningitis and aquaporin 4 (AQP4) in the mouse brain. After *Streptococcus suis* infection, the streptococcal number was calculated, and AQP4 mRNA expression in the brain was quantified at 2 and 7 days after infection. At 7 days post-infection, mice with neurological symptoms showed significantly higher *S. suis* levels in the brain than mice without neurological symptoms. AQP4 expression was significantly decreased in mice with neurological symptoms than in mice without neurological symptoms. Image analysis demonstrated that *S. suis* progressed to invade the white matter. Pathological analysis revealed that infected mouse brains had higher inflammation and neurological damage scores than uninfected mouse brains. Therefore, mice with neurological symptoms caused by streptococcal meningitis had high *S. suis* levels in the brain and reduced AQP4 expression.

Introduction

Streptococcus suis is a gram-positive, facultative anaerobic bacterium. Approximately 35 *S. suis* serotypes have been reported (De-Greeff et al. 2002). Serotype 2 is the most virulent serotype and is frequently isolated in swines and humans. Several virulence-associated genes have been reported, such as muramidase-released protein (Smith et al. 1992), extracellular protein factors (Smith et al. 1993), and suilysin (Lun et al. 2003). The primary infection routes of *S. suis* are infection of a wound (Gottschalk et al. 2010) or the gut from consuming raw pork (Nakayama et al. 2013). Several descriptions of human clinical manifestations of *S. suis* infection have been published (Werheim et al. 2009). According to our epidemiological study, approximately 20% of patients had diarrhoea and altered consciousness, and hearing loss is a unique characteristic of this infection (Kerdsin et al. 2011). Although mouse models have been used as infectious experimental models to examine the responses of mice to cytokines and chemokines produced during *S. suis* infection (Dominguez-Punaro et al. 2008), the relationship between streptococcal meningitis and brain pathology has not been adequately studied. Almost all studies have only mentioned meningitis after detecting *S. suis* in the brain. Aquaporins (AQPs) are membrane proteins involved in water transport within the body (Verkman et al. 2000). AQP4 has been identified in the brain and participates in water homeostasis (Iacovetta et al. 2012). The astrocyte plasma membrane domains that ensheath the cerebral microvessels are enriched in AQP4 water channels, which are strongly implicated in brain oedema (Papadopoulos et al. 2007, Tang et al. 2013). However, the relationship between AQP4 expression and streptococcal meningitis remains unknown. Our aim was to clarify the relationship between streptococcal meningitis and AQP4 in the mouse brain.

The *S. suis* 31533 strain was provided by the National Institute of Animal Health in Japan. Todd-Hewitt broth (Difco Laboratories, Detroit, MI, USA) was used for streptococcal cultures. A total of 22 females at 7- to 9-week-old of specific pathogen-free A/J mice (SLC, Shizuoka, Japan) were acclimated under standard laboratory conditions and provided free access to rodent chow and water. Then, 500 μL of streptococcal suspension (5.0×10^7 colony forming units (CFU) of *S. suis*) or the control solution (sterile

PBS) were administered to mice via intraperitoneal injection. Animals in a septic state lose the ability to maintain their body temperature, and a decrease in body temperature beyond a certain point (a decrease of 6 °C from normal body temperature) has been correlated with death in several infectious disease models (Olfert et al. 2000). Therefore, mouse body temperature was measured to determine the clinical endpoints. Animal studies were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Osaka University (Osaka, Japan), and all animal experiments were approved by Osaka University (Animal Welfare Assurance of Compliance H-21-09-0).

Three and five mice showing neurological symptoms were sacrificed at 2 and 7 days after infection, respectively, and three non-infected mice were sacrificed as a control using 100 µL of Ketalar solution, composed of 10 mL Ketalar (Daiichi-Sankyo, Tokyo, Japan) mixed with 2.2 mL of 2% Selactar (Bayer Health Care, Leverkusen, Germany). The brains were aseptically excised and transferred to 500 µL of sterile PBS in a cell strainer (BD Biosciences, San Jose, CA, USA) and homogenised using the rubber tip of a syringe bar (Terumo, Tokyo, Japan). The homogenised solution was used for RNA extraction. Serial dilutions of 10 µL of the homogenate in PBS were spread onto sheep blood agar plates and incubated at 37 °C for 24 h. Streptococcal colonies were counted and expressed as CFU g^{-1} for brain samples.

RNA was extracted as previously described (Nakayama et al. 2010). Briefly, PBS-Trizol (Invitrogen, Carlsbad, CA, USA) and chloroform (Wako, Osaka, Japan) were added after washing. After centrifugation, the supernatant was treated with 2-propanol (Wako, Osaka, Japan) and 70% ethanol (Wako). Complementary DNA (cDNA) was obtained using a Roche cDNA kit (Roche, Basel, Switzerland). Real-time polymerase chain reaction (PCR) was performed (Applied Biosystems, Warrington, UK) under the following conditions: 40 cycles of 94 °C for 20 s, 53 °C for 20 s, and 72 °C for 30 s. Two overlapping primers for aquaporin 4 (forward, 5'-CTG GAG CCA GCA TGA ATC CAG-3'; reverse, 5'-TTC TCT CTT CTC CAC GGT CA-3') and beta-actin (forward, 5'-GTC CCT CAC CCT CCC AAA AG-3'; reverse, 5'-GCT GCC TCA ACA CCT CAA CCC-3') were used in the present study. Standard DNA amounts corresponding to the target sequences are required to perform real-time PCR. This standardisation was achieved by purifying plasmid DNA containing the target sequences (Furrie et al. 2005). Briefly, cDNA from *S. suis* 31533 was amplified using a specific PCR primer pair. The product of the correct size and sequence was purified using a PCR purification kit (Qiagen, Valencia, CA, USA) and ligated into a vector using TOPO TA cloning (Invitrogen). DH5 alpha competent *Escherichia coli* cells were transformed with each ligated vector, and positive colonies were selected. The plasmid from each selected colony was purified using a miniprep system (Qiagen, Valencia, USA).

To clarify the localisation of *S. suis*, the brain tissue was stained and observed using a fluorescence microscope. The brain of one uninfected mouse was used as a negative control, and the brains of two mice that exhibited neurological symptoms at 7 days after infection were used for the immunostaining assays. After excision, the brain tissues were preserved in 10% neutral buffered formalin (Fujimi Pharmaceutical Company, Osaka, Japan). The brains were sectioned by the Research Foundation for Microbial Diseases at Osaka University, and the sections were embedded in paraffin. The slides were deparaffinised in xylene three times for 5 min each. The slides were immersed in 100% ethanol twice for 5

min each, and then immersed in 90% and 80% ethanol. The slides were immersed in 1 mM EDTA (pH 8.0) at 121 °C for 5 min. For immunostaining, the specimens were immersed in 5% albumin in PBS for 60 min. Rabbit anti-*S. suis* polyclonal antibody (10000×) (Statens Serum Institute, Copenhagen, Denmark) was used as the primary antibody and incubated at 37 °C for 60 min. Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) (200×) was used as a secondary antibody and incubated with the slides for 60 min. After washing, the slides were stained with haematoxylin and eosin (H&E). Finally, the aqueous mounting medium Permafluor (Thermo Fisher Scientific, Waltham, USA) was added to the specimens. All slides were examined under a fluorescence microscope. A histopathological analysis was conducted to clarify the inflammatory and neuronal damage score at 7 days post-infection, according to a previously described method (Wellmer et al. 2001). The tissue was sectioned and stained with H&E. The stained tissue was assessed using inflammatory and neuronal damage scores. The data are presented as the mean values and standard errors of the mean and were generated using Student's t-test in Figure 1a and b and a Mann-Whitney test in Figure 1(c).

After measuring the number of bacteria in the brain after infection, we detected 4.5×10^6 and 10 CFU/g in mice with neurological symptoms and 8.6×10^5 and 2.4×10^2 CFU/g in mice without neurological symptoms at 2 and 7 days after infection, respectively (Fig. 1a). There was no difference in the number of bacteria between mice with and without neurological symptoms 2 days after infection; however, the difference became apparent at 7 days after infection. We quantified AQP4 expression after infection and detected 59 and 29 copy numbers in mice with neurological symptoms and 3.7×10^3 and 1.2×10^5 copy numbers in mice without neurological symptoms at 2 and 7 days after infection, respectively (Fig. 1b). AQP4 expression was significantly decreased after infection and was significantly lower in mice with neurological symptoms than in mice without neurological symptoms at 2 and 7 days after infection.

Streptococcal localisation in the brain 7 days after infection was observed, and the bacteria were localised in the lateral ventricles in the brains of mice that exhibited neurological symptoms. No *S. suis* was detected in the brains of non-infected mice or mice without neurological symptoms (Fig. 2a). H&E staining results showed leukocyte aggregation in the lateral ventricles of mice with neurological symptoms 7 days after infection, which was consistent with the localisation of the bacteria in the lateral ventricles (Fig. 2b). The brains were analysed based on the inflammatory and neurological damage scores 7 days after infection. Pathological analysis based on H&E staining showed that mouse brains with and without neurological symptoms had inflammation and neurological damage scores, whereas the brains of uninfected mice had no scores (Table1).

Streptococcal infection can cause meningitis and neurological symptoms; however, the pathogenesis of neurological symptoms is not fully understood. When mice become infected and develop neurological symptoms, they never recover. Although almost all mice that exhibited neurological symptoms died of sepsis and bacteraemia, a few mice survived for more than 6 months following the onset of neurological symptoms.

Immunohistochemistry and histopathological analyses revealed that *S. suis* was localised to the lateral ventricles in the white matter of the brain, and lymphocyte aggregation in mice that exhibited neurological symptoms. Previous studies have reported that brain abnormalities are associated with the development of sensorineural hearing loss caused by ependymoma, which is derived from ependymal cells traversing the central nervous system (Morris et al. 2009) and may develop in response to cytomegalovirus infection (Matsuno et al. 2014). Cytomegalovirus infections in the developing brain may result in abnormalities, such as mental retardation, microcephaly, chorioretinitis, seizures, intracranial calcification, and neurological disorders, including hearing loss. These infections are commonly found in the periventricular white matter region (Moinuddin et al. 2003).

Several patients with *S. suis* exhibited a unique after effect of hearing loss (Kerdsin et al. 2011). Thus, detectable *S. suis* levels in the lateral ventricle may play an important role in the development of hearing loss. Mice that exhibited neurological symptoms harboured higher concentrations of *S. suis* in the whole brain than mice that did not show neurological signs. Therefore, high concentrations of residual *S. suis* in the brain white matter strongly correlate with the induction of neurological symptoms in the host.

We also examined AQP4 expression. AQP4 is a membrane protein involved in water transport in many fluid-transporting tissues (Niu et al. 2012, Cruz et al. 2013). Although oedema is highly related to AQP4 expression (Tang et al. 2013), the relationship between neurological damage due to infection and AQP4 is poorly understood. AQP4 expression in the brain was decreased in mice that exhibited neurological symptoms at 2 days post-infection. Although few studies have reported the relationship between AQP4 and prevention of microbial infection, water channel AQP4 partially protects the host from cerebral malaria (Promeneur et al. 2013). Moreover, the Shiga toxin released by

E. coli decreased AQP4 levels throughout the cell, which compromised the integrity of the blood-brain barrier via the activation of astrocytes (Amran et al. 2013). Based on these findings, AQP4 is a critical factor in the progression of encephalitis. In the present study, AQP4 expression was significantly higher in mice that recovered from *S. suis* infection than mice that exhibited neurological symptoms. A previous study showed that AQP4 levels were significantly higher in the cerebral cortex (grey matter) than in other parts of the brain (Han et al. 2004), and immunogold electron microscopy demonstrated that AQP4 is restricted to the glial membrane and ependymal cells (Balladh et al. 2004). Therefore, *S. suis* invaded the brain much better in mice that exhibited neurological symptoms than in symptom-free mice, and *S. suis* broke down the cells of the cerebral cortex, including AQP4. *S. suis* invaded the white matter, at which point the mice began to exhibit neurological symptoms. The present study demonstrated that *S. suis* was present in the white matter of mice that exhibited neurological symptoms. However, we could not clarify the region of white matter that was related to the induction of neurological symptoms due to *S. suis* infection, and this clarification is an important topic for future study.

In conclusion, mice that exhibited neurological symptoms also harboured high *S. suis* levels and down-regulated AQP4 levels in the brain. The image analysis demonstrated that *S. suis* progressed to invade the white matter in the brain.

Declarations

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References

Amran MY, Fujii J, Suzuki SO, Kolling GL, Villanueva SY, Kainuma M, Kobayashi H, Kameyama H, Yoshida S (2013) Investigation of encephalopathy caused by Shiga toxin 2c-producing *Escherichia coli* infection in mice. PLoS One 8:e58959. http://doi: 10.1371/journal.pone.0058959.

Balladh P, Braun A, Nedergaar M (2004) The blood-brain barrier: an overview structure, regulation, and clinical implications. Neurobiol. Dis. 16:1-13. http:// doi: 10.1016/j.nbd.2003.12.016.

Cruz NF, Ball KK, Froehner SC, Adams ME, Dienel GA (2013) Regional registration of [6-14 C] glucose metabolism during brain activation of alpha-syntrophin knockout mice. J. Neurochem. 24:10. http://doi: 10.1111/jnc.12166.

De-Greeff A, Buys H, Verhaar R, Van-Alphen L, Smith HE (2002) Distribution of environmentally regulated genes of *Streptococcus suis* serotype 2 among *S. suis* serotypes and other organisms. J Clin Microbiol 40:3261-8. https://doi.org/10.1128/jcm.40.9.3261-3268.2002

Dominguez-Punaro M, Segura M, Radzioch D, Rivest S, Gottschalk M (2008) Comparison of the susceptibilities of C57BL/6 and A/J mouse strains to *Streptococcus suis* serotype 2 infection. Infect Immun 76:3901-10. http://doi: 10.1128/IAI.00350-08

Furrie E, Macfarlane S, Kennedy A, Cumming JH, Walsh SV, O'Neil DA, Macfarlane GT (2005) Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomized controlled pilot trial. Gut 54:242-9. http://doi: 10.1136/gut.2004.044834.

Gottschalk M, Xu J, Calzas C, Segura M (2010) *Streptococcus suis*: a new emerging or an old neglected zoonotic pathogen? Future Microbiol 5:371-91. http://doi: 10.2217/fmb.10.2.

Han BC, Koh SB, Lee EY, Seong YH (2004) Regional difference of glutamate-induced swelling in cultured rat brain astrocyte, Life Sci 76:573-83. http:// doi: 10.1016/j.lfs.2004.07.016.

Iacovetta C, Rudloff E, Kirby R (2012) The role of aquaporin 4 in the brain. Vet Clin Pathol 41:32-44. http://doi: 10.1111/j.1939-165X.2011.00390.x.

Kerdsin A, Dejsirilert S, Puangpatra P, Sripakdee S, Chumla K, Boonkerd N, Polwichai P, Tanimura S, Takeuchi D, Nakayama T, Nakamura S, Akeda Y, Gottschalk M, Sawanpanyalert P, Oishi K (2011) Clinical features of *Streptococcus suis* serotype 2 infections in relation to the genotypic profiles of isolates in Thailand, Emerg Infect Dis 17:835-42. https://doi.org/10.3201/eid1705.100754.

Lun S, Perez-Casal J, Connor W, Willson PJ (2003) Role of suilysin in pathogenesis of *Streptococcus suis* capsular serotype 2. Microb Pathog 34:27-37. http://doi: 10.1016/s0882-4010(02)00192-4

Matsuo K, Morioka I, Oda M, Kobayashi Y, Nakamachi Y, Kawano S, Nagasaka M, Koda T, Yokota T, Morikawa S, Miwa A, Shibata A, Minematsu T, Inoue N, Yamada H, Iijima K (2014) Quantitative evaluation of ventricular dilatation using computed tomography in infants with congenital cytomegalovirus infection, Brain Dev. 36:10-5. http://doi: 10.1016/j.braindev.2012.12.009.

Morris EB, Li C, Khan RB, Sanford RA, Boop F, Pinlac R, Xiong X, Merchant TE (2009) Evolution of neurological impairment in pediatric infratentorial ependymoma patients. J. Neurooncol. 94 (2009) 391-8. https://doi.org/10.1007/s11060-009-9866-8.

Moinuddin A, McKinstry RC, Martin KA, Neil JJ (2003) Intracranial hemorrhage progressing to porencephaly as a result of congenitally acquired cytomegalovirus infection and illustrative report. Prenat. Diagn. 23:797-800. http:// doi: 10.1002/pd.688

Nakayama T, Takeuchi D, Matsumura T, Akeda Y, Fujinaga Y, Oishi K (2013) Alcohol consumption promotes the intestinal translocation of *Streptococcus suis* infection. Microb Pathog 65:14-20. http://doi: 10.1016/j.micpath.2013.08.006

Nakayama T, Takeuchi D, Akeda Y, Oishi K (2010) *Streptococcus suis* infection induces bacterial accumulation in the kidney. Microb Pathog 50:87-93. http://doi: 10.1016/j.micpath.2010.11.005.

Niu D, Kondo T, Nakazawa T, Kawasaki T, Yamane T, Mochizuki K, Kato Y, Matsuzaki T, Takata K, Katoh R (2012) Differential expression of aquaporins and its diagnostic utility in thyroid cancer, PLoS One 7:e40770. http://doi: 10.1371/journal.pone.0040770

Olfert E.D, Godson DL (2000) Humane endpoints for infectious disease animal models. Institute for laboratory animal research 41:99-104. http://doi: 10.1093/ilar.41.2.99.

Papadopoulos MC, Verkman AS (2007) Aquaporin-4 and brain edema. Pediatr Nephrol 22:778-84. http://doi: 10.1007/s00467-006-0411-0.

Promeneur D, Lunde LK, Amiry-Moghaddam M, Agre P (2013) Protective role of brain water channel AQP4 in murine cerebral malaria. Proc Nati Acad Sci USA 20:107-12. http:// doi: 10.1073/pnas.1220566110.

Smith HE, Vecht U, Gielkens AL, Smits MA (1992) Cloning and nucleotide sequence of the gene encoding the 136 kDa surface protein (muramidase-released protein) of *Streptococcus suis* type 2, Infect Immun 60:2361-7. https://doi.org/10.1128/IAI.60.6.2361-2367.1992

Smith HE, Reek FH, Vecht U, Gielkens AL, Smits MA (1993) Repeats in an extracellular protein of weakly pathogenic strains of *Streptococcus suis* type 2 are absent in pathogenic strains, Infect. Immun. 61:3318-26. http://doi: 10.1128/IAI.61.8.3318-3326.1993

Tang Z, Sun X, Huo G, Xie Y, Shi Q, Chen S, Wang X, Liao Z (2013) Protective effects of erythropoietin on astrocytic swelling after oxygen-glucose deprivation and reoxygenation: mediation through AQP4 expression and MAPK pathway. Neuropharmacology 67:8-15. http://doi: 10.1016/j.neuropharm.2012.10.017.

Verkman AS, Yang B, Song Y, Manley GT, Ma T (2000) Role of water channels in fluid transport studied by phenotype analysis of aquaporin knockout mice. Exp Physiol 85:233S-41S. http://doi: 10.1111/j.1469-445x.2000.tb00028.x.

Wertheim HF, Nguyen HN, Taylor W, Lien TT, Ngo HT, Nguyen TQ, Nguyen BN, Nguyen HH, Nguyen HM, Nguyen CT, Dao TT, Nguyen TV, Fox A, Farrar J, Schultsz C, Nguyen HD, Nguyen KV, Horby P (2009) *Streptococcus suis*, an important cause of adult bacterial meningitis in northern Vietnam, PLoS One 22:e5973. http://doi: 10.1371/journal.pone.0005973.

Wellmer A, Gerber J, Ragheb J, Zysk G, Kunst T, Smirnov A, Bruck W, Nau R (2001) Effect of deficiency of tumor necrosis factor alpha or both of its receptors on Streptococcus pneumonia central nervous system infection and peritonitis. Infect. Immun 69:6881-6. http://doi: 10.1128/IAI.69.11.6881-6886.2001.

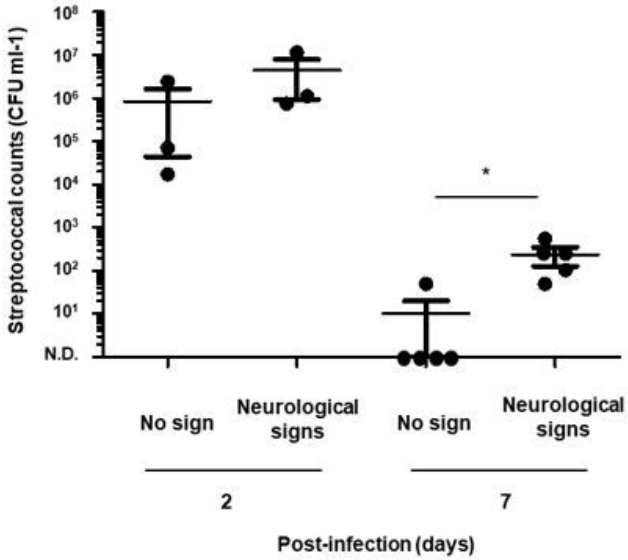
Tables

Table 1 Pathological analysis 7 days after infection

	Inflammatory score (Meningeal and temporobasal)	Neuronal damage score (Cortex)
No sign	4	2
Neurological signs	1.5	2
No infection	0	0

Figures

(a)



(b)

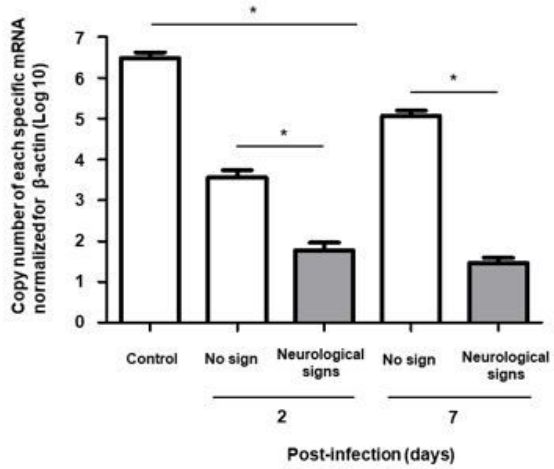


Figure 1

Influence of streptococcal number and AQP4 expression by streptococcal infection. At 2- and 7-days post-infection, the brain tissues were sampled. (a) Number of streptococcal CFUs was calculated, and (b) mRNA AQP4 expression was quantified by real-time PCR. All data were statistically analysed using Student's t-test (a) and Mann-Whitney test (b) (*$p < 0.05$).

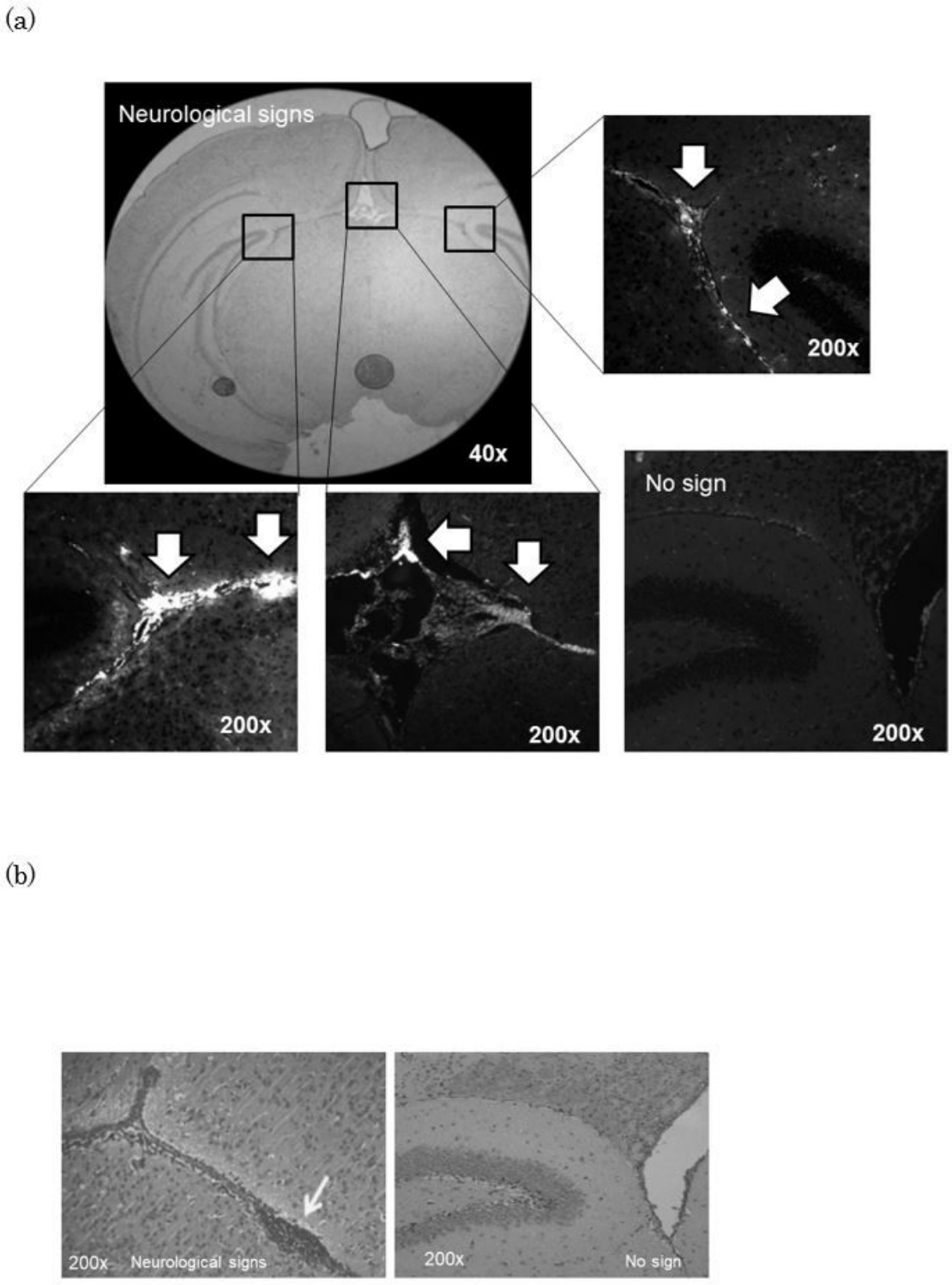


Figure 2

Image analysis of mice brains infected with S. suis. After 7 days post-infection, the brain tissues were sampled. (a) Sampled brains were subjected to immunohistochemistry, and the presence of S. suis was confirmed by fluorescence microscopy using an Alexa Fluor 488-conjugated antibody. (b) Histopathological analysis of the brain at 7 days post-infection. The white arrow shows that the lymphocyte aggregation in the lateral ventricle was confirmed in mice with neurological symptoms.