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Dermal mast cells reduce progressive tissue necrosis caused by subcutaneous infection with *Streptococcus pyogenes* in mice

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A single subcutaneous (s.c.) infection with 1×10^7 c.f.u. GAS472, a group A streptococcus (GAS) serotype M1 strain isolated from the blood of a patient suffering from streptococcal toxic shock syndrome, led to severe damage of striated muscle layers in the feet of mast cell (MC)-deficient WBB6F$_1$-*Kit*^{W}/*Kit*$^{W\text{-}v}$ (*W*/*W*^{v}) mice 72 h after infection. In contrast, no damage was recognized in striated muscle layers in the feet of the control WBB6F$_1$-*Kit*$^{+/+}$ (+/+) mice 72 h after infection. In addition, adoptively transferred MCs reduced progressive tissue necrosis of the feet of *W*/*W*^{v} mice after infection. However, there was no significant difference in the mortality rates between the *W*/*W*^{v} and +/+ mice, or between the human CD46-expressing transgenic (Tg) mouse bone marrow-derived cultured MC-reconstituted *W*/*W*^{v} and non-Tg mouse bone marrow-derived cultured MC-reconstituted *W*/*W*^{v} mice after infection. Consequently, although MCs can help to reduce the severity of necrosis of the feet caused by s.c. infection with GAS472, such reduction of tissue necrosis scarcely improves the mortality rates of these mice. Moreover, human CD46 does not play a crucial role in the MC-mediated innate immune defence against GAS infection.

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INTRODUCTION

Group A streptococcus (GAS) is a bacterium often found in the throat and on the skin of humans, and most people infected with GAS have no symptoms of illness. Some GAS infections result in relatively mild illnesses such as 'strep throat' or impetigo. However, GAS can occasionally cause severe and even life-threatening diseases. Severe GAS diseases may occur when the bacteria invade parts of the body where they are usually not found, such as the blood, muscle or lungs. These infections are termed invasive GAS diseases. Two of the most severe, but least common, forms of invasive GAS disease are necrotizing fasciitis (NF) and streptococcal toxic shock syndrome. NF (occasionally described by the media as being caused by the 'flesh-eating' bacterium) is defined pathologically by a deep spreading infection of the subcutaneous (s.c.) tissue that results in the progressive destruction of fascia and fat, with relative sparing of the skeletal muscle (Bisno & Stevens, 1996; Filbin *et al.*, 2009; Fustes-Morales *et al.*, 2002; Leitch *et al.*, 2000).

In humans, the skin is able to activate the innate immune response in reaction to GAS, which involves antimicrobial peptides functioning on the effector side of the immune system (Steinstraesser *et al.*, 2008). In a recent study, mast cell (MC) cathelicidin LL-37, a cationic antibacterial peptide, was shown to protect mice against skin infection with GAS (Di Nardo *et al.*, 2008). Generally, cutaneous MCs are known to contribute to the pathology of various skin disorders, including allergic and autoimmune dermatoses (Leslie, 2007). Although MCs have mainly been studied in the setting of allergic disease, they can also be activated as part of the innate immune response to pathogens (Galli *et al.*, 2005; Marshall, 2004).

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Abbreviations: GAS, group A streptococcus; MC, mast cell; NF, necrotizing fasciitis; s.c., subcutaneous; Tg, transgenic.

Most of the *in vivo* work on MC function in antibacterial host defence is done using models of bacterial infections of the peritoneum; however, recent studies have shown that MCs also play this role in other anatomical sites such as the middle ear (Ebmeyer *et al.*, 2005), the lung (Xu *et al.*, 2006) and skin (Siebenhaar *et al.*, 2007). The skin plays an important role as a barrier against exogenous hazards, including microbes and physical stimuli such as UV light. MCs contribute to this barrier function and can act as sentinels in the skin, helping to limit or even prevent the damage that results from these environmental threats (Metz *et al.*, 2008). We therefore aimed to investigate whether dermal MCs can protect mice from systemic infection with GAS472 by preventing NF. In this study, we provide evidence to suggest that the innate immune defence by MCs against GAS is limited to a reduction of the severity of localized tissue necrosis.

METHODS

Mice. The human CD46-expressing transgenic (Tg) mice were donated by Dr J. P. Atkinson of Washington University. The C57BL/6 mice employed as non-Tg control mice were obtained from Charles River Japan, where they were established as described previously (Matsui *et al.*, 2009). MC development *in vivo* is highly dependent on the cytokine kit ligand/stem cell factor on the surface of mesenchymal cells and its tyrosine kinase receptor c-Kit/CD117 on the surface of MC-committed progenitors. Signalling through the c-kit results in the translocation of microphthalmia transcription factor into the nucleus. WBB6F$_1$-*Kit*^{W}/*Kit*$^{W\text{-}v}$ (*W/W*^{v}) mice are MC-deficient secondary to a point mutation in the intracellular tyrosine kinase domain, which makes their MCs and progenitors less responsive to the c-kit ligand (Kitamura *et al.*, 2007; Thakurdas *et al.*, 2007). C57BL/6-*Kit*$^{W\text{-}sh}$/*Kit*$^{W\text{-}sh}$ (Wsh$^{-/-}$) (H-2^{b}) mice represent another MC-deficient model for studies of MC functions *in vivo* (Galli *et al.*, 2005). In this study, the *W/W*^{v} and control WBB6F$_1$-*Kit*$^{+/+}$ (+/+) mice were obtained from SLC Japan. All mice were bred at the animal facility at the Kitasato Institute, and all mouse experiments were performed in accordance with institutional guidelines under an approved protocol.

Bacteria. GAS472 (β-haemolytic GAS serotype M1 strain) was isolated from the blood of a patient suffering from streptococcal toxic shock syndrome in Japan in 2006. GAS472 was grown in Todd–Hewitt broth containing 0.2 % (w/v) yeast extract (Difco and BBL) in 5 % CO_2 at 37 °C without shaking (Matsui *et al.*, 2009).

Infection. The expression of many pathogenic traits of GAS has been shown to depend on the growth phase (Miyoshi-Akiyama *et al.*, 2003). Thus, the hind footpads of 12-week-old female mice were subcutaneously infected with 1×10^7 c.f.u. GAS472 in stationary growth phase. After s.c. infection, the survival rates were observed every 24 h until 336 h post-infection, and the numbers of viable bacteria in the liver, spleen and popliteal lymph nodes were determined by plating onto sheep blood agar (Eguchi *et al.*, 2007; Kodama *et al.*, 2005).

Macroscopic and microscopic observations. Macroscopic images were obtained with a digital camera (D80; Nikon). For histological examination, a portion of each footpad was fixed with 4 % (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and then embedded in paraffin. Tissue sections approximately 5 μm thick were prepared and mounted on glass slides. The slides were stained with haematoxylin and eosin or Giemsa. Alternatively, another portion of each footpad was also fixed with Zamboni's fixative (2 %, v/v, paraformaldehyde and 15 %, v/v, saturated picric acid solution in 0.1 M phosphate buffer, pH 7.3) for at least 8 h at 4 °C (Stefanini *et al.*, 1967). The fixed tissue samples were embedded in optimum cutting temperature compound and rapidly frozen by isopentane chilled with liquid nitrogen. The 4 μm cryosections were stained with toluidine blue.

Selective MC reconstitution of *W/W*^{v} mice. Bone marrow cells were harvested from both 7-week-old female CD46 Tg and C57BL/6 mice and cultured in complete RPMI 1640 supplemented with 10 % fetal bovine serum, 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1}, 50 μM 2-mercaptethanol and 4 ng interleukin-3 ml^{-1} (PeProtec EC). Bone marrow-derived cultured MCs were used at >99 % purity, as determined by flow cytometric analysis using FcεRI^{+} (eBioscience) and *c-kit*^{high} (Beckman Coulter) (Furuta *et al.*, 2006). Bone marrow-derived cultured MCs from CD46 Tg mice were also used to determine the expression of human CD46 by flow cytometric analysis using FITC-conjugated anti-human CD46 mAb (BD Biosciences). At the time of reconstitution, bone marrow cells from *W/W*^{v} mice were treated with a combination of anti-Thy 1 mAb and guinea pig serum to remove any contamination of T cells. The T cell-depleted bone marrow cells (2×10^6 per mouse) and bone marrow-derived cultured MCs (5×10^6 per mouse) from either CD46 Tg or C57BL/6 mice were injected intravenously and intraperitoneally, respectively, into X-ray-irradiated (4.5 Gy ×2; MBR-1520R-3, Hitachi Medical) 6-week-old female *W/W*^{v} mice. The bone marrow-derived cultured MC-reconstituted *W/W*^{v} mice were housed for 6 weeks before infection with GAS472.

Statistics. The survival was analysed using a Kaplan–Meier log rank test. Significant differences between the mean ± SD values of different groups were examined using a two-tailed unpaired Student's *t*-test. A *P*-value of <0.05 was regarded as statistically significant.

RESULTS AND DISCUSSION

CD46 Tg mice exhibit degranulation of MCs in the footpad skin sections after s.c. infection with GAS472

Human CD46 was first recognized as an epidermal keratinocyte receptor for the M protein of GAS (Okada *et al.*, 1995). These findings were followed by the discovery that engagement of human CD46 and $\alpha5\beta1$ integrin by GAS was required for the efficient invasion of epithelial cells (Rezcallah *et al.*, 2005). Meanwhile, mammalian skin is composed of three layers: the epidermis, dermis and hypodermis (s.c. layer or superficial fascia). The dermis is tightly connected to the epidermis by a basement membrane, and the hypodermis lies beneath the dermis. In addition, muscles are separated from the underlying hypodermis. In our recent study, histological observation of the footpad skin sections of CD46 Tg mice subcutaneously infected with GAS472 revealed that acute inflammation developed in the hypodermis at 6 h post-infection, exfoliation of the epidermis with intracellular oedema and haemorrhaging developed in the dermis at 24 h post-infection, and necrosis of the striated muscle layers developed at 48 h post-infection (Matsui *et al.*, 2009). In the present study, toluidine blue staining of the footpad

skin sections revealed the presence of dermal MCs in uninfected CD46 Tg mice (Fig. 1a). Then, following the s.c. infection with GAS472, MC degranulation appeared to begin and to progress gradually (Fig. 1b, c, d). These findings provide histological evidence that the extracellular traps by MCs are formed after s.c. infection with GAS472. Therefore, it is suggested that the dermal MCs in CD46 Tg mice might be specialized in many ways to contribute to the innate immune defence system against s.c. infection with GAS472.

MCs suppress the GAS472-induced destruction of the muscle layer

Quite a few reports have previously shown that MC deficiency results in a markedly elevated susceptibility to a host of different bacterial and parasitic infections in addition to GAS infection. For example, MC-deficient mice are much more susceptible to infection caused by *Salmonella enterica* serovar Typhimurium (Chatterjea *et al.*, 2005), *Mycoplasma pneumoniae* (Xu *et al.*, 2006), *Citrobacter rodentium* (Wei *et al.*, 2005), *Helicobacter felis* (Velin *et al.*, 2005), *Haemophilus influenzae* (Ebmeyer *et al.*, 2005), *Listeria monocytogenes* (Gekara & Weiss, 2008), *Pseudomonas aeruginosa* (Siebenhaar *et al.*, 2007), *Leishmania major* (Maurer *et al.*, 2006) and *Plasmodium berghei* (Furuta *et al.*, 2006). In order to clarify the role of MCs in mice during GAS infection, MC-deficient W/W^v and their control +/+ mice were subcutaneously infected with GAS472. Although the necrotizing lesions were observed in the feet of both W/W^v (Fig. 2b) and +/+ (Fig. 2f) mice at 72 h post-infection, the destruction of muscle layers in the skin sections was recognized in W/W^v mice (Fig. 2c) but not in +/+ mice (Fig. 2g). In contrast, large numbers of inflammatory cells were visible around the muscle layers of the footpad skin sections of +/+ mice (Fig. 2g), whereas only small numbers of inflammatory cells were recognized around the destructed muscle layers in the footpad skin sections of W/W^v mice (Fig. 2c). Much of this variation would be associated with differences in bacterial amounts within tissues. Indeed, although clusters of streptococci were found at the destructed muscle layers in W/W^v mice (Fig. 2d), bacteria were detected only at the dermis in +/+ mice (Fig. 2h) at this stage. In addition, W/W^v mice had a significantly higher number of viable bacteria in the samples of the liver, spleen and popliteal lymph nodes compared with +/+ mice 72 h after s.c. infection with GAS472 (Fig. 3). These findings suggest that the severity of necrosis in the s.c. tissues and muscle layers of the feet of mice during GAS infection correlated with the number of disseminated bacteria in the involved tissues.

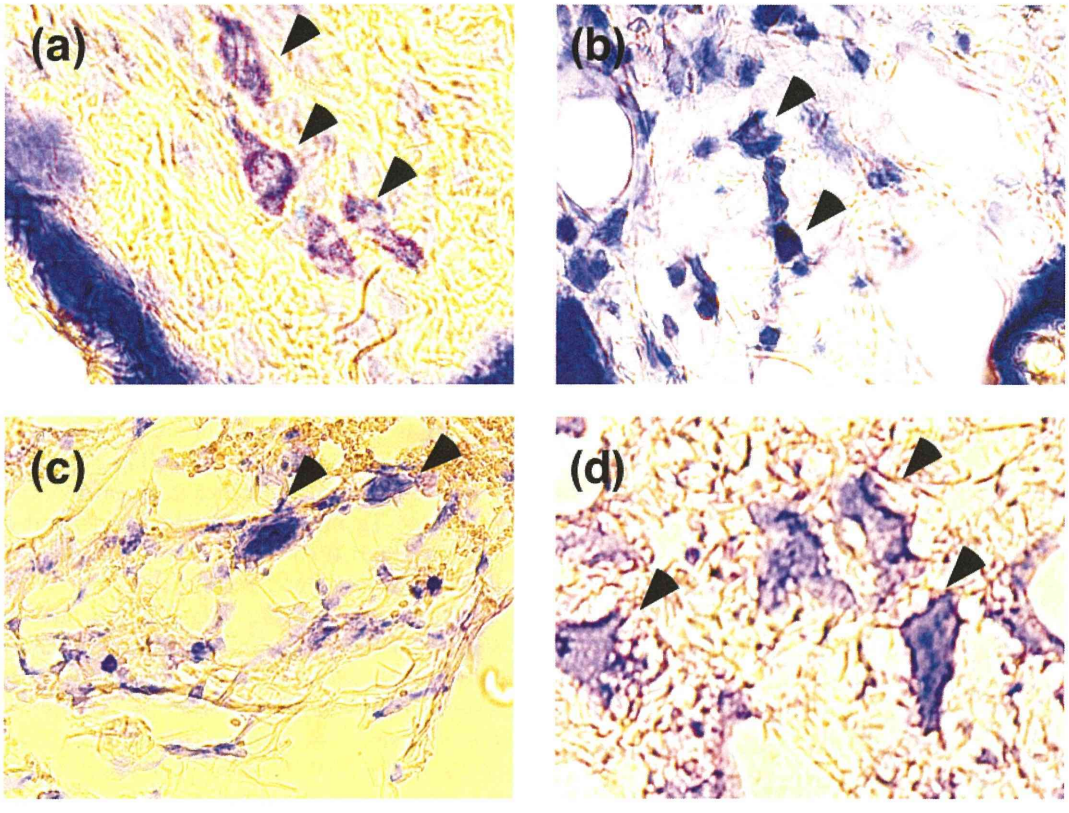

Fig. 1. Footpad skin sections of CD46 Tg mice. Toluidine blue-stained footpad skin sections from uninfected CD46 Tg (a) and GAS472-infected CD46 Tg (b, c, d) mice at 24 h (b), 48 h (c) or 72 h (d) after s.c. infection with 1×10^7 c.f.u. GAS472. Arrowheads indicate the MCs. Original magnifications, ×200.

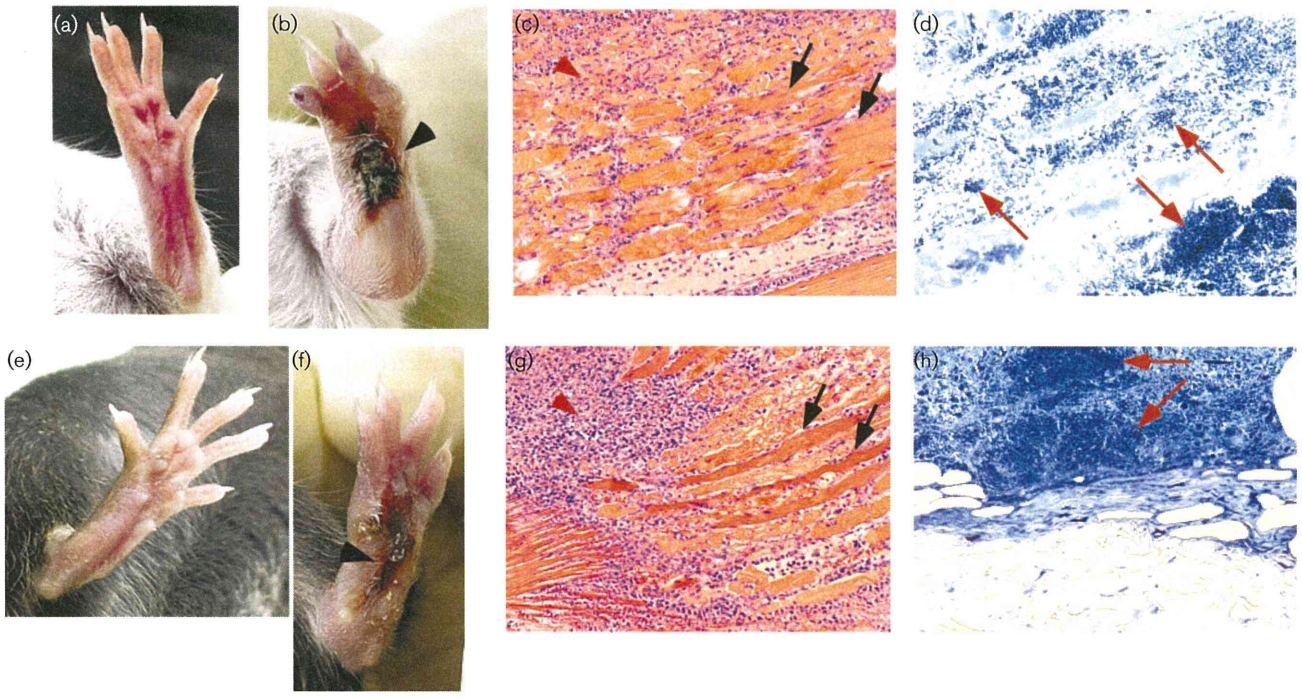

Fig. 2. Representative appearance of the hind feet with histological observations of *W/W*^{v} and +/+ mice. Macroscopic images of the feet from uninfected control *W/W*^{v} (a) and +/+ (e) mice, followed by the feet from infected *W/W*^{v} (b) and +/+ (f) mice at 72 h after s.c. infection with 1×10^{7} c.f.u. GAS472. Haematoxylin and eosin-stained (c, g) or Giemsa-stained (d, h) footpad skin sections of s.c. infected *W/W*^{v} (b, c, d) and +/+ (f, g, h) mice are also shown. The black arrowheads indicate the necrotizing lesions (b, f). The red arrowheads and black arrows indicate the inflammatory cells and the striated muscle layers, respectively (c, g). The red arrows indicate the clusters of streptococci (d, h). Original magnifications, ×100 (c, g), ×200 (h) and ×400 (d).

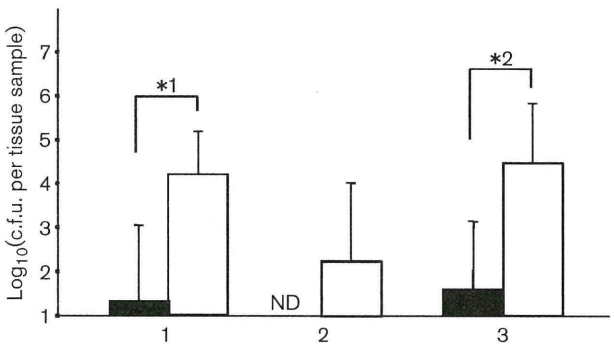

Fig. 3. Bacterial counts in tissues of *W/W*^{v} and +/+ mice. *W/W*^{v} (open columns) and +/+ (closed columns) mice were subcutaneously infected with 1×10^{7} c.f.u. GAS472. At 72 h post-infection, the numbers of viable bacteria in the liver (1), spleen (2) and popliteal lymph node (3) samples were determined by plating. Data represent the mean value of the number of bacteria per tissue sample ± SD. *1, P=0.009; *2, P=0.017 (*W/W*^{v} mice vs +/+ mice). ND, Not detected. Each group has six mice.

MCs do not contribute to the survival rates of mice after s.c. infection with GAS472

Based on the fact that *W/W*^{v} mice had a significantly higher number of viable bacteria in the deep tissues compared with +/+ mice (Fig. 3), a clear difference in mortality rates would be expected between *W/W*^{v} and +/+ mice after s.c. infection with GAS472. However, we found that there was no significant difference in the mortality rates between *W/W*^{v} and +/+ mice (Fig. 4a). In addition, there was no significant difference in the mortality rates between the CD46 Tg mouse bone marrow-derived cultured MC-reconstituted *W/W*^{v} and non-Tg mouse bone marrow-derived cultured MC-reconstituted *W/W*^{v} mice after s.c. infection with GAS472 (Fig. 4b). In fact, there were no significant differences in mortality between any two of the four groups. Meanwhile, as compared with the necrotizing lesions of surviving mice after s.c. infection with GAS472, the necrosis of the skin, s.c. tissue, muscle and even bones of the feet of *W/W*^{v} mice progressed more rapidly after infection. In contrast, NF developed only partially in the feet of non-Tg mouse bone marrow-derived cultured MC-reconstituted *W/W*^{v} mice after infection (Fig. 5). Apparently, the adoptively transferred MCs reduced progressive tissue necrosis in the feet of *W/W*^{v} mice after

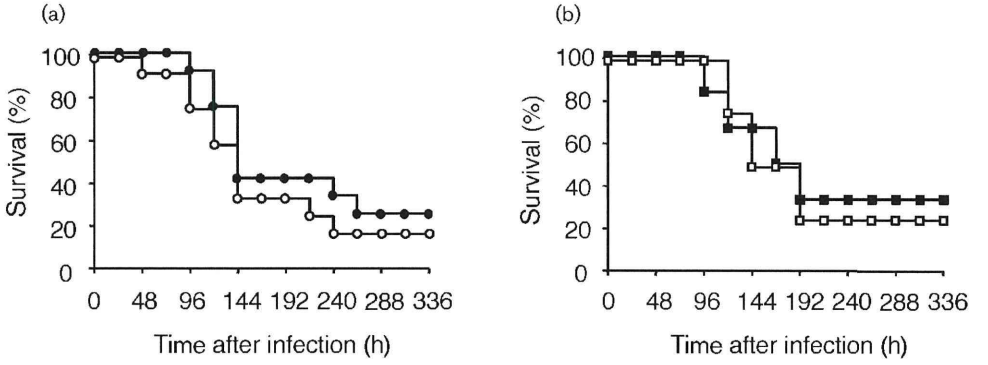

Fig. 4. Comparison of survival rates between *W/W*^{v} and +/+ mice or between the CD46 Tg mouse bone marrow-derived cultured MC-reconstituted *W/W*^{v} and non-Tg mouse bone marrow-derived cultured MC-reconstituted *W/W*^{v} mice. The mice infected subcutaneously with 1×10^{7} c.f.u. GAS472 were monitored every 24 h for survival during the 336 h study. ○, *W/W*^{v} mice (a; $n=12$); ●, +/+ mice (a; $n=12$); □, CD46 Tg mouse bone marrow-derived cultured MC-reconstituted *W/W*^{v} mice (b; $n=12$); ■, C57BL/6 bone marrow-derived cultured MC-reconstituted *W/W*^{v} mice (b; $n=12$). (a) $P=0.47$ (+/+ mice vs *W/W*^{v} mice); (b) $P=0.91$ (CD46 Tg mouse bone marrow-derived cultured MC-reconstituted *W/W*^{v} vs C57BL/6 bone marrow-derived cultured MC-reconstituted *W/W*^{v} mice).

s.c. infection with GAS472. Therefore, it was concluded that even though MCs can reduce progressive tissue necrosis, they do not participate in the improvement of host mortality due to GAS infection. Moreover, human CD46 does not play a crucial role in the MC-mediated innate immune defence during GAS infection.

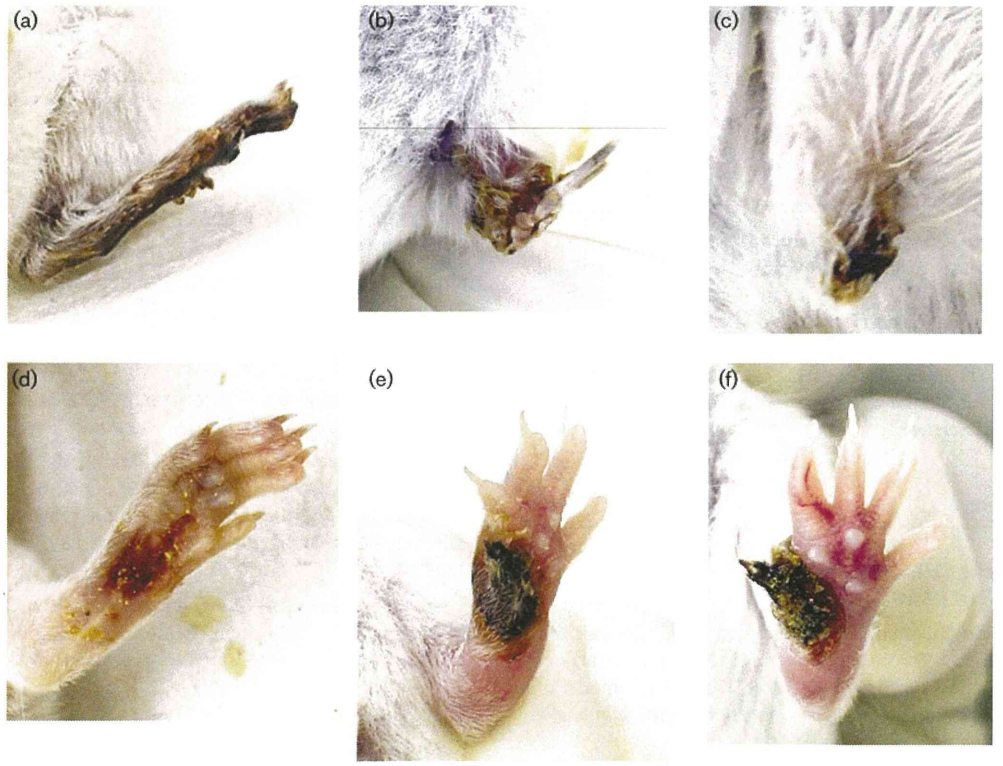

Fig. 5. Representative appearance of hind foot lesions of *W/W*^{v} and non-Tg mouse bone marrow-derived cultured MC-reconstituted *W/W*^{v} mice. Macroscopic observations of the feet of *W/W*^{v} (a, b, c) and non-Tg mouse bone marrow-derived cultured MC-reconstituted *W/W*^{v} (d, e, f) mice at 72 h (d), 120 h (a), 168 h (b, e) or 336 h (c, f) post-infection with 1×10^{7} c.f.u. GAS472.

Local MCs play a limited role in innate immunity

In human patients, NF due to GAS infection is defined pathologically by a deep-seated infection of the s.c. tissue that results in the progressive destruction of fascia and fat, with relative sparing of the skeletal muscle (Bisno & Stevens, 1996; Filbin *et al.*, 2009; Fustes-Morales *et al.*, 2002; Leitch *et al.*, 2000). In the present study, GAS472 infection of W/W^v mice resulted in typical rhabdomyolysis in the muscle layers at 72 h post-infection (Fig. 2c), followed by the progressive destruction of skin, s.c. tissue, muscle and even bone (Fig. 5a, b and c), which indicates that the infected W/W^v mice developed progressive and widespread tissue necrosis in their feet that was involved in myonecrosis and osteonecrosis, as well as NF. In the present murine experimental model of GAS infection, we showed that MCs play a key role in the reduction of progressive tissue necrosis caused by s.c. infection with GAS472 (Figs 2 and 5). Even so, a vital question still remains: namely, why was there no significant difference in the mortality rates between the W/W^v and +/+ mice (Fig. 4a)? MCs are preferentially located at sites with higher risks of bacterial infection. MC numbers within the whole skin tissue are highest in the most superficial skin layers and lowest in the subcutis, and they increase with the distance of the anatomical site from the body centre (Maurer & Metz, 2005). The MC-mediated protection against GAS infection should be linked to MC degranulation at an early stage after infection (Fig. 1). In our view, MCs are able to trap and kill bacteria, but their relatively low numbers (e.g. less than 5 % of skin cells) and their finite mobility prevent them from playing a major role in the direct bactericidal action in deep tissues.

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ORIGINAL ARTICLE

Invasive *Streptococcus pneumoniae* infections in children in Kamikawa and Soya subprefecture, Hokkaido, Japan, 2000–2010, before the introduction of the 7-valent pneumococcal conjugate vaccine

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Abstract We evaluated 103 cases of invasive pneumococcal disease (IPD) encountered in 99 children (two developed the disease twice and one, three times) treated in the northern district of Hokkaido (Kamikawa and Soya subprefecture) from April 2000 until March 2010, before the introduction of the 7-valent pneumococcal conjugate vaccine. The main diseases were as follows: pneumonia, 54 cases (52.9%); occult bacteremia, 34 cases (33.3%); meningitis, five cases (4.9%). There were 42 cases during the first half of the study period (from April 2000 to March 2005) and 61 during the second half (from April 2005 to March 2010). The IPD morbidity rate for the 10-year period was 41.3 per 100,000 population in children <5 years and 79.2 per 100,000 population in children <2 years. Serotype analysis of the 77 strains was performed. The most frequent serotype isolated was 6B (31.2%), followed by 23F (14.3%), 19F (13.0%), 9V (7.8%), 6A (7.8%), and 14 (3.9%). The number of strains that could potentially be covered by heptavalent pneumococcal conjugate vaccine was 55 (71.4%), and the number of strains that could potentially be covered by 13-valent pneumococcal conjugate vaccine was 64 (83.1%). Analysis of penicillin-binding protein (*PBP*) genes was performed of the 82 strains. The percentages of resistant bacteria caused by *PBP* gene mutations were 42.7% (35 strains) for gPRSP, 48.8% for gPISP (40 strains), and 8.5% for gPSSP (7 strains).

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Introduction

Streptococcus pneumoniae is an important causative pathogen of meningitis, sepsis, pneumonia, otitis media, etc., in children. Diseases such as meningitis, bacteremia, pneumonia, and arthritis, in which *S. pneumoniae* is detected from normally aseptic sites, i.e., cerebrospinal fluid, blood, pleural effusion, and synovial fluid, respectively, are referred to as invasive pneumococcal disease (IPD).

Numerous reports from various concerning invasive pneumococcal disease (IPD) and its morbidity rates have been published [1–5]; however, reports from Japan are scarce. Recently, the heptavalent pneumococcal conjugate vaccine (PCV-7) against 7 serotypes of *S. pneumoniae* (4, 6B, 9V, 14, 18C, 19F, and 23F), was introduced in many countries, and with its introduction, the incidence of IPD has reportedly decreased. In the near future, a 13-valent pneumococcal conjugate vaccine (PCV-13), against 13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F), is proposed to be introduced worldwide. In Japan, however, even PCV-7 was just approved in March 2010 for voluntary vaccination, and it will take time for the vaccination rate to rise sufficiently to produce a decrease in IPD morbidity rate.

To assess the efficacy of PCV-7 in Japan, it is essential to know IPD morbidity rate before the vaccine's introduction. For this purpose, we asked nine hospitals with pediatric inpatient facilities in the northern district of Hokkaido (Kamikawa and Soya subprefectures) to conduct a survey to determine the IPD morbidity rate and to collect

the bacterial strains isolated each year. Herein, we report the IPD morbidity rate, the diseases encountered, the serotypes of the clinical isolates, and the status of resistance for the 10 years from April 2000 until March 2010.

Patients and methods

The northern district of Hokkaido has an area of about 13,902 km^2 and a population of about 610,000. It has nine medical institutions with pediatric inpatient facilities. In this report, we summarize the ages of IPD patients and the resultant diseases treated in these facilities during the 10-year period from April 2000 until March 2010. Personal information was anonymous, and it was impossible to connect patient data. Serotypes and the status of the presence of penicillin-binding protein (*PBP*) resistance genes of *S. pneumoniae* were examined. We compared the results from the first half of the study period (from April 2000 to March 2005) with those from the second half of the study period (from April 2005 to March 2010).

PBP genes were analyzed using a polymerase chain reaction (PCR) kit (Wakunaga Pharmaceutical Co., Ltd. Tokyo, Japan) containing reagents for detecting genes responsible for the development of penicillin-resistant *S. pneumoniae* [6]. Based on the results, we classified the samples as penicillin-resistant *S. pneumoniae* (PRSP), penicillin-intermediate-resistant *S. pneumoniae* (PISP), and penicillin-susceptible *S. pneumoniae* (PSSP) according to the classification of Ubukata et al. [6]. Genotype determination was indicated by adding "g" to designations, such as gPSSP, gPISP, and gPRSP. Serotypes were assessed with pneumococcal antisera (Statens Serum Institute, Copenhagen, Denmark).

Morbidity rates were calculated as the number of patients per 100,000 population per annum. The estimated population of the district was based on results of the National Census in 2000 for the first half of the study period (<5 years, 25,597; <2 years, 10,078) and on the National Census in 2005 for the second half of the study period (<5 years, 24,021; <2 years, 9,341).

The statistical significance of the results were assessed using Mann–Whitney's test for numerics and the chi-square test for ratios; statistical analyses were conducted using StatMate III for Macintosh (ATMS), with the significance level defined at $p < 0.05$.

Results

We analyzed 103 cases of IPD encountered in 99 children (two children developed the disease twice and one child three times) during the 10-year period. There were no children with immunoglobulin disorders or asplenia, including among those who developed IPD more than once.

There were 42 cases during the first half of the study period and 61 during the second half. IPD morbidities for the 10-year period were 41.3 per 100,000 population in children <5 years and 79.2 per 100,000 population in children <2 years. Morbidity per 100,000 population in children <5 years rose from 32.0 in the first half of the study period to 48.8 in the second half. Similarly, that of children <2 years rose from 61.5 in the first half of the study period to 89.9 in the second half.

Age distribution of children with IPD in the first half of the study period was 0 years, 14 (33.3%); 1 year, 18 (42.9%); 2 years, 5 cases (11.9%); 3 years, 1 case (2.4%); 4 years, 3 cases (7.1%); 6 years, 1 case (2.4%). Similarly, age distribution of children with IPD in the second half of the study period was 0 years, 9 cases (14.8%); 1 year, 33 cases (54.1%); 2 years, 7 cases (11.5%); 3 years, 9 cases (14.8%); 4 years, 1 case (1.6%); 5 years, 1 case (1.6%); 6 year, 1 case (1.6%). About half of the affected children were 1 year old in both the first and second half of the study period, and the morbidity rate decreased with age. No significant difference was noted between age distributions during the first and second half of the study period.

Diseases were pneumonia, 54 cases (52.9%); occult bacteremia, 34 cases (33.3%); meningitis, 5 cases (4.9%); pharyngitis, 3 cases (2.9%), otitis media, 3 cases (2.9%); arthritis, 2 cases (1.8%); encephalopathy, 1 case (0.9%); orbital phlegmon, 1 case (0.9%). Thus, pneumonia accounted for about half of all cases. Morbidity rates per 100,000 population by disease in children <5 years were pneumonia, 43.0; occult bacteremia, 27.2; meningitis, 4.1. Comparisons of morbidity rates of various types of IPD between the first half and second half of the study period (shown as first half vs. second half) were pneumonia, 15 (35.7%) vs 37 cases (60.7%); occult bacteremia, 15 (35.7%) vs 19 cases (31.1%); meningitis, 3 (7.1%) vs 2 cases (3.3%). Morbidity rate of pneumonia increased significantly from the first to the second half of the study period. At the end of treatment, all children were alive without sequelae.

Serotype analysis was performed of the 30 and 47 strains isolated during the first and second half of the study period, respectively. The most frequent serotype isolated was 6B (24 strains, 31.2%), followed by 23F (11 strains, 14.3%), 19F (10 strains, 13.0%), 9V (6 strains, 7.8%), 6A (6 strains, 7.8%), and 14 (3 strains, 3.9%). The number of strains that could potentially be covered by PCV-7 was 55 (71.4%), and the number that could potentially be covered by PCV-13 was 64 (83.1%). No difference in the frequency of isolation between the first and second half of the study period was noted for any of the strains (Fig. 1).

PBP gene analysis was performed of the 35 and 47 strains isolated during the first and second half of the study

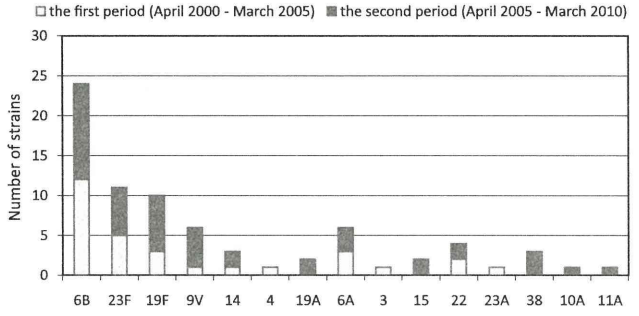

Fig. 1 Serotype distribution of *Streptococcus pneumoniae* isolated from children with invasive pneumococcal disease

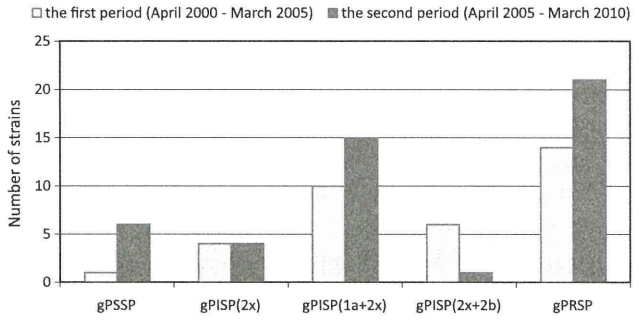

Fig. 2 Resistant pattern of *Streptococcus pneumoniae* isolated from children with invasive pneumococcal disease. *PSSP* penicillin susceptible *Streptococcus pneumoniae*, *PISP* penicillin intermediate resistant *Streptococcus pneumoniae*, *PRSP:PISP* penicillin resistant *Streptococcus pneumoniae*

period, respectively. The percentages of resistant bacteria isolated during the study period that were caused by the mutations were 42.7% (35 strains) for gPRSP, 48.8% for gPISP (40 strains), and 8.5% for gPSSP (7 strains). Among the gPISP strains, the most frequent mutation was the 2× type, which was seen in 30.5% (25 strains). The results of a similar analysis for the 31 strains isolated during the first half of the study period were 40.0% for gPRSP (14 strains), 57.1% for gPISP (20 strains), and 2.9% for gPSSP (1 strain). Similarly, analysis of the 46 strains isolated during the second half of the study period revealed 44.7% for gPRSP (21 strains), 42.6% for gPISP (20 strains), and 12.8% for gPSSP (6 strains) (Fig. 2).

Discussion

Reports on morbidity rates of IPD from Japan are scarce. Among the very few reports, Ishiwada et al. [7] conducted a survey on the annual morbidity rates from 2003 to 2005 in Chiba Prefecture and reported morbidity rates per 100,000 population of between 19.5 and 23.8 in children <2 years and between 12.6 and 13.8 in children <5 years. The results of their study and those of our study reported here appear to be substantially different. The reason for these differences is unclear. We consider that to clarify the reason, a systematic surveillance system needs to be devised to correctly evaluate PCV efficacy.

For further comparisons, we introduce the reader to some reports from the USA and Europe regarding IPD morbidity rates before PCV-7 implementation. Robinson et al. [8], from the USA, reported that the morbidity rate in children <2 years per 100,000 population between 1995 and 1998 was 166.9: occult bacteremia, 120.3; meningitis, 7.5; pneumonia, 24.0. Ispahani et al. [9], from the UK, examined 266 children with IPD between 1980 and 1999 and reported morbidity rates per 100,000 population of 37.8 in children <2 years and 20.0 <5 years : meningitis, 32%; pneumonia, 31%; occult bacteremia, 30%. Pineda et al. [10], from Spain, examined 112 patients with IPD between 1990 and 2000 and reported morbidity rates per 100,000 population of 76.4 in children <2 years and 45 in children <4 years: occult bacteremia, 66 cases (58.9%); pneumonia, 34 cases (30.4%); meningitis, 10 cases (8.9%); arthritis, 2 cases (1.8%). Morbidities in other western European countries were similar [5], and they were nearly the same or slightly lower than the morbidity rates determined in our study.

Pineda et al. [10] inferred that the reason for the lower morbidity rates in western European countries compared with those in the USA might not be attributable to the regional differences in the frequency of blood cultures but might, instead, be explained by the fact that if pediatricians routinely performed blood cultures when treating infants with fever, the morbidity rate would be increased. In other words, they considered that in countries or regions where blood cultures were rarely performed for infants with fever, the estimated morbidity rates were not lower but were simply detected less frequently.

Chiba et al. [11] examined the serotypes of 191 strains of *S. pneumoniae* isolated from children with IPD in Japan during 2006 and 2007 and reported that the most frequent was 6B (43 strains), followed by 19F (27 strains), 14 (25 strains), and 23F (23 strains), and that the potential coverages by the PCV-7 and PCV-13 vaccines were 75.4 and 93.7%, respectively. These coverages were slightly higher than those obtained in our study. Johnson et al. [12] reviewed 169 reports on IPD morbidity rates from around the world and examined the potential coverages by the two types of vaccines. They reported that coverages by PCV-7 and PCV-13, respectively, were: Asia 52% and 77%, Europe 72% and 88%, North America 82% and 88%; Central and South America 58% and 82%, Oceania 68% and 79%, and Africa 49% and 77%. When compared with the results of Chiba et al. [11] and our results, these results represent higher PCV-7 coverage and similar PCV-13 coverage in Asia. The reason may be that in all of Asia

except Japan, there are relatively many strains of serotypes 1 and 5 among the strains of IPD-causing *S. pneumoniae*.

Many reports indicate decreased IPD morbidity rate in the USA [2, 8] and Europe [4, 5], which is attributable to the widespread use of PCV-7. On the other hand, the IPD morbidity rate caused by *S. pneumoniae* of serotypes 19A, 7F, etc., which are not covered by PCV-7, has been increasing [13, 14]. We believe that in Japan, attention should be paid to changes in the prevalent serotypes with the increase of PCV-7 vaccination.

As for resistance status, Chiba et al. [11] reported the following: gPRSP, 46.1%; gPISP (pbp2x), 19.9%; gPISP, (pbp1a + 2x) 12.6%; gPISP (pbp2x + 2b), 6.2%; gPISP (pbp2b), 1.0%; gPSSP 14.1%. These results were almost the same as those of our study, and thus we consider that the above detection frequencies of individual resistant strains may be representative of those in Japan.

Although our survey was small in terms of population, we believe that the results were reasonable, considering other reports from Japan and overseas. We propose to trace the effect of PCV-7 in the future.

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Genotypic Profile of *Streptococcus suis* Serotype 2 and Clinical Features of Infection in Humans, Thailand

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To examine associations between clinical features of *Streptococcus suis* serotype 2 infections in humans in Thailand and genotypic profiles of isolates, we conducted a retrospective study during 2006–2008. Of 165 patients for whom bacterial cultures of blood, cerebrospinal fluid, or both were positive for *S. suis* serotype 2, the major multilocus sequence types (STs) found were ST1 (62.4%) and ST104 (25.5%); the latter is unique to Thailand. Clinical features were examined for 158 patients. Infections were sporadic; case-fatality rate for adults was 9.5%, primarily in northern Thailand. Disease incidence peaked during the rainy season. Disease was classified as meningitis (58.9%) or nonmeningitis (41.1%, and included sepsis [35.4%] and others [5.7%]). Although ST1 strains were significantly associated with the meningitis category ($p<0.0001$), ST104 strains were significantly associated with the nonmeningitis category ($p<0.0001$). The ST1 and ST104 strains are capable of causing sepsis, but only the ST1 strains commonly cause meningitis.

Streptococcus suis, an emerging zoonotic pathogen, causes invasive infections in persons who are in close contact with infected pigs or contaminated pork-derived products (*1*). On the basis of capsular polysaccharides, 33 serotypes of *S. suis* have now been identified. Of these, serotype 2 is the most prevalent type in humans infected with this pathogen (*1,2*). Since the largest outbreak of human *S. suis* infection in 2005, in Sichuan Province, People's Republic of China (*3*), this disease has been increasingly recognized worldwide. The numbers of reported cases, especially in persons from Southeast Asian countries, have increased dramatically during past few years (*4*).

In Thailand, at least 300 cases of *S. suis* infection in humans have been reported (*5–11*). Although an outbreak of *S. suis* infections was confirmed in Phayao Province during May 2007 (*9*), most cases in humans occur sporadically and are primarily located in the northern region of this country (*6–11*). A relatively low incidence of cases with *S. suis* serotype 14 has also been reported in this region (*12*). Although previous studies have reported high frequencies (59.0%–88.7%) of *S. suis* infections in persons in this area who ate raw pork products (*8–11*), the pathogenesis of this disease, including routes of transmission, is unclear.

The major clinical manifestations of the disease are bacterial meningitis and sepsis, but other manifestations have been reported (*1,4, 8,10,13*). Most cases of bacterial meningitis can be attributed to the hematogenic spread of invasive bacteria, but how circulating bacteria cross the blood–cerebrospinal fluid (CSF) barrier and cause meningitis is not clear (*14,15*). Furthermore, the overall clinical features of this disease have not been extensively and comprehensively investigated in Southeast Asian countries.

A variety of virulence factors associated with *S. suis* have been reported (*16–20*), but none have been proven to be essential for the host defense of this disease, except

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the capsular polysaccharide (*19*). In serotype 2 isolates obtained during a previous outbreak in Sichuan, China, an ≈89-kb DNA fragment, which has been associated with a pathogenicity island (89K PAI), was identified (*21*). The 89K PAI fragment encodes a 2-compartment signal transduction system, SalK-SalR, which is required for full virulence (*22*).

We report the results of a retrospective study of the clinical features of 158 cases of human infection with *S. suis* serotype 2 and the molecular epidemiology of 165 *S. suis* serotype 2 isolates. The study objective was to demonstrate associations between the clinical features of disease caused by *S. suis* serotype 2 in persons in Thailand and the genotypic profiles of the isolates. The study was reviewed and approved by the Ethics Committees of Research Institute for Microbial Diseases, Osaka University, and conducted according to the principles expressed in the Declaration of Helsinki.

Methods

Isolate Identification

From January 2006 through August 2008, a total of 1,154 unidentified streptococcal isolates from blood or CSF were collected from hospitals in all 76 provinces of Thailand. Biochemical testing of these isolates, using API Strep (bioMérieux, Durham, NC, USA) and *S. suis*–specific and *S. suis* serotype 2– or 1/2–specific PCR (*12,23*), confirmed 165 isolates from 34 hospitals in 25 provinces as *S. suis*. The final serotype of all strains was confirmed by coagglutination tests that used rabbit antiserum (Statens Serum Institute, Copenhagen, Denmark).

Genotypic Profiles of Isolates

Multilocus sequence type (MLST) testing was performed as described by King et al. (*24*), with a modification for *mutS* as described by Rehm et al (*25*). MLST alleles and the resulting sequence type (ST) were assigned by using the *S. suis* MLST database (http://ssuis.mlst.net). eBURST was used to identify the clonal complexes for these 165 serotype 2 strains within *S. suis*, and the overall structure of the population was obtained through the MLST database (*26*). Virulence-associated genes (VAG), including extracellular released protein factor (*epf*), muramidase-released protein (*mrp*), and suilysin (*sly*), and variants of *mrp* or *epf* were determined by PCR as described by Silva et al. (27), with minor modifications. Presence of the 89K PAI fragment was determined by PCR as reported by Chen et al (*21*). Pulsed-field gel electrophoresis (PFGE) was performed as described (*28*), and the pulsotypes were assigned to clusters of isolates with >80% similarity from the dendrogram. The dendrogram representing the genetic relationships between the representative pulsotypes from 165 *S. suis* serotype 2 strains was drawn by using the Cluster 3.0 software program and examined by using the TreeView program as described (*12,29*).

Clinical Features of Cases

Of the 165 patients whose culture results were positive for *S. suis* serotype 2, medical records for 158 were retrospectively reviewed by physicians at local hospitals in Thailand. Medical records for the remaining 7 patients were not available. The clinical manifestations were mostly divided into 2 categories: meningitis and nonmeningitis. The meningitis category involved confirmed meningitis, bacteremic meningitis, and probable meningitis. All patients in the meningitis category had typical meningeal signs, such as neck stiffness, and acute disease onset. Although bacteremic meningitis was defined as a case in which both CSF and blood cultures were positive, confirmed meningitis was defined as a case with a positive CSF culture only, and probable meningitis was defined as a case with a positive blood culture only. The nonmeningitis category included the clinical manifestations of sepsis and sepsis with focal signs other than meningitis (septic arthritis or spondylodiscitis, infective endocarditis, and bacteremic pneumonia). Sepsis was defined as systemic inflammatory response syndrome and a positive blood culture (*30*), and septic arthritis or septic spondylodiscitis was defined as described (*31*). Diagnosis of infectious endocarditis was based on the Duke criteria (*32*). Septic shock was also defined as described (*33*).

Statistical Analyses

Comparisons of the clinical characteristics between fatal and nonfatal cases were analyzed by using the χ^2 test or Fisher exact test with Stata version 10.0 software (StataCorp, College Station, TX, USA). Patient ages and periods of hospital admission were tested for normality of the distribution using the Kolmogorov-Smirnov test and were compared by using the Student *t* test with SPSS version 11.0 software (SPSS Inc., Chicago, IL, USA). Data were considered significant at $p<0.05$.

Results

Genotypic Profiles of Isolates

Of the 165 *S. suis* serotype 2 isolates, 123 were isolated from blood and 42 from CSF. eBURST analysis based on MLST enabled classification of these strains into 4 ST complexes: the ST1, ST27, ST29, and ST104 complexes (Table 1). ST126, a novel ST, has a single locus variant from ST1. The largest cluster of 89K PAI–carrying strains was ST1 (n = 81, 49.1%), which had the *epf*+/*sly*+/*mrp*+ genotype; these strains were isolated from blood and CSF. Another large cluster of non-89K PAI–carrying strains was

Table 1. Genotypic profiles of 165 clinical isolates of *Streptococcus suis* serotype 2, Thailand, January 2006–August 2008*

ST complex	ST	VAG†	Isolation site	89K PAI +	89K PAI –	No. (%) strains
1	1	*epf–/sly+/mrp+*	Blood	1	0	103 (62.4)
		epf+/sly+/mrp+	Blood	52	13	
			CSF	29	5	
		epf+/sly+/mrp^{s}	Blood	0	1	
			CSF	0	2	
	126	*epf+/sly+/mrp+*	Blood	1	0	3 (1.8)
			CSF	2	0	
27	28	*epf–/sly–/mrp+*	Blood	0	1	3 (1.8)
			CSF	0	2	
29	25	*epf–/sly–/mrp**	Blood	8	0	11 (6.7)
		epf–/sly–/mrp+	Blood	3	0	
	103	*epf–/sly–/mrp**	Blood	2	0	3 (1.8)
		epf–/sly–/mrp+	Blood	1	0	
104	104	*epf–/sly+/mrp–*	Blood	3	38	42 (25.5)
			CSF	0	1	
Total no. strains	NA	NA	NA	102	63	165 (100)

*ST, sequence type; VAG, virulence-associated gene; 89K PAI, an ≈89-kb pathogenicity island; CSF, cerebrospinal fluid; NA, not applicable.
†*mrp*^{s} and *mrp** are *mrp* variants that produce ≈750-bp and ≈1,800-bp fragments, respectively, by PCR (*23,34*).

ST104, which had the *epf–/sly+/mrp–* genotype (n = 39, 23.6%); most of these strains (n = 38) were isolated only from blood. ST103, ST104, and ST126 were found only in isolates from humans in Thailand.

PFGE of Isolates

Of the 165 serotype 2 strains, PFGE analyses identified 20 pulsotypes (Figure 1, panel A). Analysis of the dendrogram for these 20 pulsotypes revealed at least 16 clusters (I to XVI) (Figure 1, panel B). Although 5 pulsotypes of A were identified for the ST1 and ST126 strains, 2 major pulsotypes (A [n = 32] and A1 [n = 43]), A1 (n = 43), and A4 (n = 3) were grouped in 1 cluster. Pulsotype A2 (n = 21), which consisted of ST1 strains lacking the 89K PAI fragment, was classified into a distinguished cluster. PFGE showed diverse DNA patterns for strains ST25 and ST103. ST25 strains were classified into 5 clusters of I, II, III, IV, and VIII. ST103 strains were classified into 3 clusters of VI, XIV, and XV. Three ST28 strains lacking 89K PAI exhibited the unique DNA pattern of pulsotype D; these were classified into cluster XVI. Although 4 pulsotypes (H, H1, H2, and H3) were identified for ST104 strains, 2 major pulsotypes (H [n = 29] and H1 [n = 11]) in ST104 strains were classified into cluster VII. Collectively, clusters X and XI for ST1 and ST126 strains and cluster VII for ST104 strains accounted for the major 3 clusters found for cases in Thailand.

Geographic and Seasonal Distribution

Of the 165 isolates, 136 (82.4%) were from the northern region, 19 (11.5%) from the central region, 7 (4.2%) from the northeast region, and 3 (1.8%) from the eastern region (Table 2; Figure 2, panel A). No strains were isolated from the southern region. The dates of isolation suggest that human cases occur more frequently during the rainy season, June–August of each year (Figure 2, panel B).

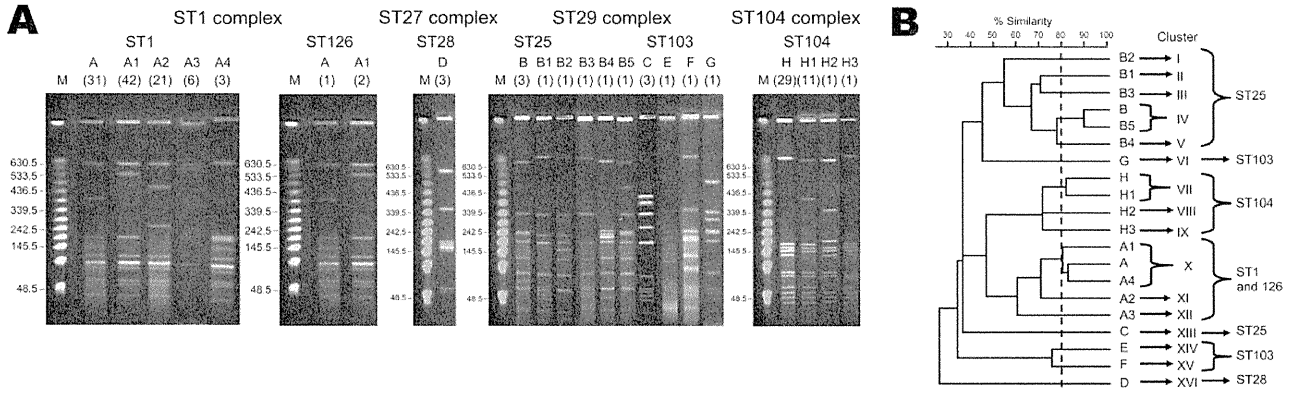

Figure 1. A) Pulsed-field gel electrophoresis profiles of 165 human isolates of *Streptococcus suis* serotype 2, after *Sma*I digestion. Numbers of isolates are indicated in parentheses below pulsotype numbers. B) Dendrogram generated from the pulsed-field gel electrophoresis profiles. ST, sequence type.

Table 2. Distribution of sequence types of 165 clinical isolates of *Streptococcus suis* serotype 2, by region, Thailand

Sequence type	North	Northeast	East	Central	South
1	85	6	1	11	0
25	11	0	0	0	0
28	3	0	0	0	0
103	1	0	1	1	0
104	33	1	1	7	0
126	3	0	0	0	0
Total	136	7	3	19	0

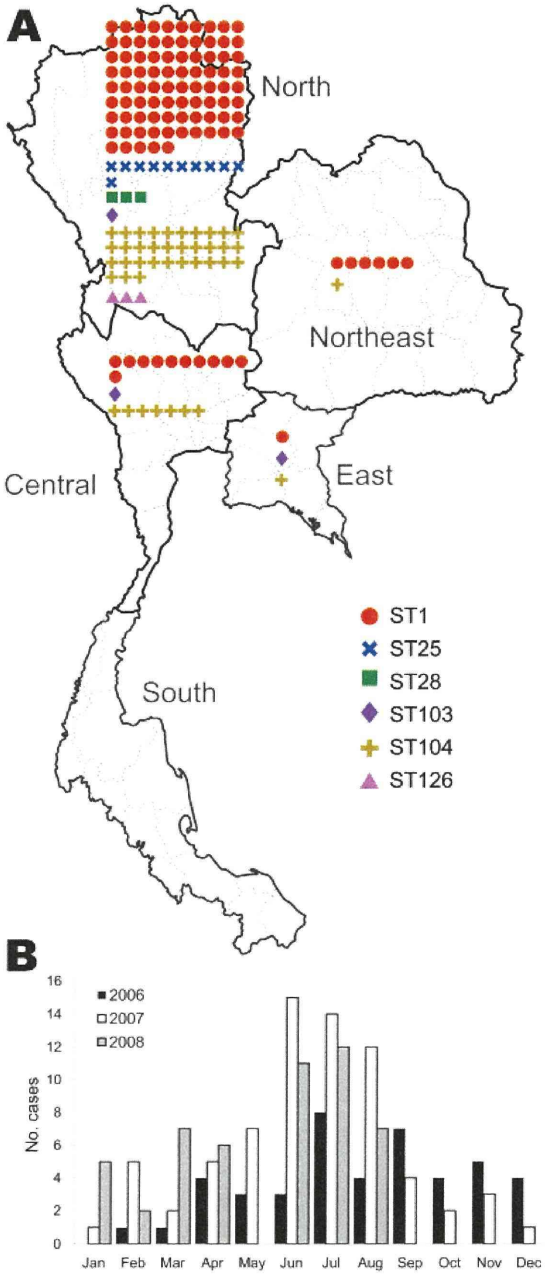

Figure 2. Distribution and sequence types (STs) of 165 human isolates of *Streptococcus suis* serotype 2, January 2006–August 2008, Thailand. A) Regions of isolation; B) monthly distribution of isolations.

Clinical Features of Cases

The clinical features of the 158 human cases of *S. suis* serotype 2 infection are summarized in Table 3. The median age (range) of the 155 patients for whom age was known was 55.0 (18–93) years; 72.8% were male. No cases in children were identified in this study. All 158 patients had been hospitalized; median duration (range) of hospitalization for the 158 patients was 11 (1–45) days; 15 (9.5%) patients died. No significant differences were found between the fatal and nonfatal cases with respect to patient age or period of admission.

The meningitis category (n = 93) included 22 cases of confirmed meningitis, 44 cases of bacteremic meningitis, and 27 cases of probable meningitis (Figure 3). The nonmeningitis category (n = 65) included sepsis with focal signs other than meningitis (n = 9) and sepsis (n = 56). Sepsis with focal signs other than meningitis included septic arthritis (n = 5), infective endocarditis (n = 3), and bacteremic pneumonia (n = 1). Of the 15 fatal cases, 8 were assigned to the meningitis category (probable meningitis [n = 6], meningitis [n = 1], bacteremic meningitis [n = 1]), 6 cases were sepsis, and 1 case was infective endocarditis (Table 3). Although the cases of bacteremic meningitis were significantly associated with a nonfatal outcome ($p = 0.043$), the probable meningitis cases were significantly associated with a fatal outcome ($p = 0.013$). The combined frequencies for the recent consumption of raw pork products and exposure to pigs were 39.9%. None of the clinical signs or possible risk factors, including recent exposure to pigs or raw pork products, or alcohol abuse, was significantly associated with a fatal outcome. Of the 158 patients, 154 parenterally received antimicrobial drugs, such as ceftriaxone, and data concerning antimicrobial drug treatment were not available for 4. Corticosteroids, such as dexamethasone, were used for only 4 patients.

Clinical Features and Genotype Profiles

The distributions of STs for the 158 human isolates for the meningitis and nonmeningitis categories are shown in Table 4. Although the ST1 strains were significantly associated with the meningitis category ($p<0.0001$), the ST104 strains were significantly associated with the nonmeningitis category ($p<0.0001$). The VAG profile of *epf+/sly+/mrp+*, which was dominant in the ST1 strains,

Table 3. Demographic and clinical features of 158 human cases of *Streptococcus suis* serotype 2 infections, Thailand, January 2006–August 2008*

Characteristic	All, n = 158	Fatal, n = 15; 9.5%	Nonfatal, n = 143; 90.5%	p value
Demographic				
Male sex, %	72.8	66.7	73.4	0.386
Mean (median) age, y†	56.6 (55.0)	53.9 (52.5)	57.0 (56.0)	0.264
Period of admission, d, mean (median)	12.5 (11)	10.1 (6)	12.9 (12)	0.737
Meningitis category, no. (%) cases				
Confirmed meningitis	22 (13.9)	1 (6.7)	21 (14.7)	0.348
Bacteremic meningitis	44 (27.8)	1 (6.7)	43 (30.1)‡	0.043
Probable meningitis	27 (17.1)	6 (40.0)	21 (14.7)§	0.013
Nonmeningitis category, no. (%) cases				
Septic arthritis	5 (3.2)	0	5 (3.2)	1
Infective endocarditis	3 (1.9)	1 (6.7)	2 (1.4)	0.905
Bacteremic pneumonia	1 (0.6)	0	1 (0.7)	1
Sepsis	56 (35.4)	6 (40.0)	50 (35.0)	0.698
Signs and symptoms, no. (%) cases				
Diarrhea	28 (17.1)	5 (33.3)	23 (16.1)	0.1
Hearing loss	34 (21.5)	4 (26.7)	30 (21.0)	0.409
Altered consciousness	35 (22.2)	4 (26.7)	31 (21.7)	0.434
Shock	9 (5.7)	2 (13.3)	7 (4.9)	0.205
Possible risk factors, no. (%) cases				
Recent consumption of raw pork products	52 (32.9)	5 (33.3)	47 (32.9)	0.589
Recent exposure to pigs	11 (7.0)	2 (13.3)	9 (6.3)	0.28
Alcohol abuse	33 (21.0)	5 (33.3)	28 (19.6)	0.178

*Statistical analyses were performed by using the χ^2 or Fisher exact test.
†Ages were not available for 3 patients.
‡One case of bacteremic meningitis was associated with pneumonia.
§Two cases of probable meningitis were associated with spondylodiscitis.

was also significantly associated with the meningitis category (p<0.0001). The VAG profile of *epf–/sly+/mrp–*, which was observed only in the ST104 strains, was also significantly associated with the nonmeningitis category (p<0.0001). Because the largest cluster of 89K PAI–carrying strains was associated with the VAG profile of *epf+/sly+/mrp+*, the presence of 89K PAI was also significantly associated with the meningitis category (p<0.0001). None of the genotypic profiles that included STs, VAG, and presence of 89K PAI were significantly associated with fatal or nonfatal outcomes (data not shown).

Discussion

Our finding that isolated *S. suis* serotype 2 strains peaked during the rainy season of 2006–2008 confirmed conclusions reached in previous small-scale studies

Table 4. Genotypic features of *Streptococcus suis* serotype 2 as risk factor for meningitis*

Feature	Clinical category, no. (%) strains			p value
	All, n = 158	Meningitis, n = 93	Nonmeningitis, n = 65	
Sequence type				
1	98 (62.0)	73 (78.5)	25 (38.5)	<0.0001†
104	40 (25.3)	6 (6.5)	34 (52.3)	<0.0001‡
25	11 (7.0)	7 (7.5)	4 (6.2)	0.478
28	3 (1.9)	2 (2.2)	1 (1.5)	0.632
103	3 (1.9)	2 (2.2)	1 (1.5)	0.655
126	3 (1.9)	2 (2.2)	0	0.201
VAG profile				
epf+/sly+/mrp+	97 (61.4)	72 (79.6)	25 (35.4)	<0.0001†
epf+/sly+/mrp^s	3 (25.3)	3 (3.2)	0 (0)	0.201
epf–/sly+/mrp–	40 (25.3)	6 (6.5)	34 (52.3)	<0.0001‡
*epf– /sly–/mrp**	10 (6.3)	6 (6.5)	4(6.2)	0.607
epf–/sly–/mrp+	7 (4.4)	5 (5.3)	2 (3.1)	0.392
epf–/sly+/mrp+	1 (1.0)	1 (1.1)	0 (0)	1
89K PAI profile, 89K PAI+	98 (62.0)	70 (75.3)	28 (43.1)	<0.0001†

*Statistical analyses were performed by using the χ^2 or Fisher exact test. VAG, virulence-associated gene; 89K PAI, ≈89-kb pathogenicity island.
†Significant association with the meningitis category.
‡Significant association with the nonmeningitis category.

conducted in northern Vietnam and Hong Kong (*35,36*). The predominant distribution of these isolates in northern Thailand is also in accordance with previous reports (*6–11*). However, why no human cases were identified in southern Thailand remains uncertain. A recent study from Hong Kong reported heavy contamination of *S. suis* in raw pork meat at local supermarkets or wet markets; therefore, a hot and humid climate may facilitate the growth of *S. suis* in raw pork products in those markets (*37*) and increase the risk for *S. suis* infections in humans in northern Thailand. The finding of no cases in children suggests that the routes of transmission are associated with adult behavior.

A recent study from northern Thailand, based on 20 human isolates collected during 1998–2002, reported that the most common isolates of *S. suis* serotype 2 were ST25 (40%), followed by ST1 (15%) and ST103 (15%) (*34*). By contrast, the MLST and PFGE results in this study clearly demonstrated that ST1 strains with major pulsotypes of A, A1 and A2, and ST104 with major pulsotypes of H and H1 were currently circulating in the same region of Thailand during 2006–2008. Collectively, these data suggest dynamic replacement of STs from ST25 to ST1 and ST104 among serotype 2 strains during recent years in this region.

Although *S. suis* serotype 2 has been reported to be the most frequent cause of bacterial meningitis in adults in Vietnam (*13,35*), other clinical manifestations, such as sepsis and infectious endocarditis, have also been found to be common in Thailand (*6,8,11*). Of the 158 human cases in the study reported here, ≈60% were assigned to the meningitis category and ≈35% were sepsis. Other clinical manifestations, including infective endocarditis, were rare. The findings reported here demonstrate significant associations between the ST1 strains and the meningitis category and between the ST104 strains and the nonmeningitis category. These findings indicate that both the ST1 and ST104 strains cause bacteremia and sepsis but that the ST1 strains are more likely to cross the blood–CSF barrier and subsequently result in meningitis. Because ≈80% of the cases in the meningitis category were caused by strains with ST1, as evidenced by a VAG profile of *epf*+/*sly*+/*mrp*+ and 89K PAI, these genotypic profiles of *S. suis* serotype 2 may favor bacterial survival and multiplication in the bloodstream, which would result in high levels of bacteremia, crossing of the blood–CSF barrier, and invasion of the meninges and the central nervous system (*15*). Our PFGE data showed that the pulsotype A1 found in serotype 2 strains with ST1 was identical to pulsotype 11 of serotype 2 strains with ST1 from Vietnam and pulsotype I of the serotype 2 strains with ST1 from Hong Kong (*13,28*). These isolates from Vietnam and Hong Kong were associated with a VAG profile of *epf*+/*sly*+/*mrp*+, and the strains from Vietnam were also the cause of meningitis in adults. A unique DNA pattern of pulsotype D, classified into cluster XVI, was found for 3 strains with ST28 isolated from nonfatal cases in this study. Previous studies also reported 1 nonfatal case caused by the ST28 strain from Thailand and Japan (*34,38*).

Associations for bacteremic meningitis cases with nonfatal outcomes and probable meningitis cases with fatal outcomes contrasted strikingly in this study. Of 6 fatal cases of probable meningitis, 2 were caused by ST1, 2 by ST25, and 2 by ST104 strains. The extent to which the virulence of each ST strain contributed to these deaths remains uncertain. Another possible explanation may be a frequent involvement of critically ill patients, for whom lumbar puncture was not possible; these patients had probable meningitis and typical meningeal signs, acute disease onset, and positive blood culture only.

Because the clinical charts were retrospectively reviewed and the etiologic diagnosis of *S. suis* infection might not have been readily reported to the attending physicians during the hospitalization of the patients in this study, the extent of investigations of clinical manifestations, possible risk factors, and causes of death might have been limited. Because different physicians were involved in the assessment of different patients in this study, the possibility of misdiagnosis for clinical categories cannot be completely excluded even though meningeal signs and acute disease onset are clinical indicators of meningitis.

In conclusion, this study of the clinical features of 158 cases of *S. suis* seotype 2 infection in humans in Thailand showed that the disease occurs sporadically in adults and results in a mortality rate of ≈9.5%; the major clinical manifestations include meningitis and sepsis. MLST analyses of 165 isolates from humans indicated that the major STs were ST1 followed by ST104. Although both ST1 and ST104 strains cause sepsis, it is likely that only the ST1 strain causes meningitis. Further studies are needed to elucidate the pathogenesis of the human *S. suis* infections that are prevalent in Southeast Asian countries.

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