Study of Pathogenicity of *Streptococcus suis* Type 2 in SPF Pigs using Immunohistopathology

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Abstract: *Streptococcus suis* serotype 2 (*S. suis 2*) is an important zoonotic pathogen that can cause severe disease and even death in both humans and swine. However, currently less knowledge is available about them. Therefore, the aim of this study was to investigate pathogenicity, localization and dynamic distribution of *S. suis 2* in tissues and organs of infected SPF pigs. Thirty 6wk old specific pathogen free (SPF) pigs were randomly divided into experimental groups (including 20 pigs) injected with either the Sichuan strain ZY05719 of *S. suis 2* (20 pigs) or aseptic medium (control, 10 pigs). The animals were sacrificed 12h, 1d, 2d, 3d, 4d, 5d, 6d, 7d, 8d, 10d after injection. Samples were collected from different tissues including tonsil, lymphoid node, liver, spleen, heart, lung, and brain. Samples were frozen and then sections were prepared for immunofluorescence and bacteria isolation studies. This study revealed that *S. suis 2* are mainly located in tonsil crypts, where they could be readily detected soon after 12h of infection. The fluorescent cell-like bacterial particles could also be seen in the marginal zone and periarterial lymphoid sheath of spleen, the germinal center and cortex of lymph node, the hepatic sinusoid of liver, interstitium of lung; furthermore, bacteria were also present in the meninges of the brain where their level fluctuated over the post-injection period of observation. However, overall dynamics of bacteria did not substantially change in the various parenchymatous organs such as liver, kidney, lung and spleen, where the number of bacteria rose steadily for 3 days following infection and declined after the 6th day post-infection. And clear-cut associations were detected between pathogens and histological lesions or histopathological diagnoses in most tissues and organs. These results provide significant amount of knowledge to investigate pathogenicity of *S. suis 2* in pigs.

Keywords: *Streptococcus suis* type 2, pathogenicity, immunofluorescence, localization, dynamic distribution.

INTRODUCTION

*Streptococcus suis* is known as a major bacterial pathogen of swine [1, 2]. Among the 35 described serotypes, serotype 2 is most commonly associated with the disease. As a zoonotic agent, *S. suis 2* causes meningitis, septicaemia, endocarditis, arthritis, and septic shock in pigs [3-5]. Two large-scale human outbreaks of *S. suis 2* infection occurred in China in 1998 and 2005, causing extensive concerns about the human health worldwide [6]. It is thought that the most important virulence factors in *S. suis 2* are the capsular polysaccharides and virulence-related proteins including muramidase-released protein, extracellular protein factor, hemolysin (suilysin) and so on [7].

The pathogenesis of *S. suis 2* has not yet been studied thoroughly, and currently the dynamic distribution and localization of *S. suis 2* in vivo is still unclear. Therefore, the aims of the present study were to elucidate the dynamic distribution and localization of *S. suis 2*, and to associate these findings with the histopathological diagnosis, in order to broaden the understanding of the pathogenesis of *S. suis 2*.

MATERIALS AND METHODS

Bacterial Strains

*S. suis 2* strains ZY05719 were provided by Professor Lu Chengping (College of Veterinary Medicine, Nanjing Agricultural University).

Experimental Animals

Six-week-old specific-pathogen-free (SPF) Bama minipigs, weighing 4-5kg, were provided by the laboratory of Zoonosis and comparative medicine of the school of agricultural and biology, Shanghai Jiao Tong University. All piglets were negative for antibodies to classical swine fever, porcine reproductive and respiratory syndrome (PRRS), foot-and-mouth disease virus, Porcine circovirus type 2(PCV2), IgB and IgE of herpes virus, H1N1 of swine influenza, Mycoplasma hyopneumoniae, Toxoplasma, brucellosis, Porcine epidemic encephalitis B and Porcine Parvovirus. The study protocol was approved by Animal Care and Use Committee (ACUC) of Shanghai Jiaotong University.

Prior to the experimental *S. suis 2* challenge, all the piglets were negative for *S. suis 2*-specific antibodies, as determined by an ELISA (KHB, Shanghai, China).
Reagents and Instruments

The working concentration of goat anti-pig IgG enzyme-labelled antibody (Shanghai Ruiji biotechnology company, Shanghai, China) was 1:2000; FITC (fluorescein isothiocyanate) was purchased from Merck (Germany) and DEAE52 cellulose was from Pierce (USA); the fluorescence microscope used was a Nr.323778 Rejchert (Austria), the freezing microtome was from Leica (Germany), the DG-3022A ELISA plate reader was from Nanjing electron tube factory (Nanjing, China), whereas 40-pore polystyrenes microtitration plates were purchased from Shanghai plastic products company (Shanghai, China).

Preparation and Characterization of the FITC-Conjugated Antibody

Preparation and Purification of S. suis 2 antisera

S. suis 2 -positive sera were prepared from two SPF pigs immunized with activated whole cell bacteria (2 mL of 1x10^9 CFU/mL each) 3 times at 2-week intervals. One week after the last injection, blood was collected from the jugular veins and the serum separated, purified, and stored at -20 °C. The level of antibody in the prepared antiserum was determined by ELISA.

On part (v:v) of PBS and a half part (v:v) of saturated ammonium sulphate were added to the S. suis 2 antiserum and incubated for 30 min at RT followed by centrifugation for 20 min at 3000 rpm, and the precipitate was discarded. One part (v:v) of PBS and two parts (v:v) of saturated ammonium sulphate were added to supernatant, incubated for 30 min at RT, centrifuged for 10 min at 10000 rpm, and the process was repeated three times. The precipitate was redissolved in PBS and dialyzed against distilled water overnight followed by dialysis against PBS for 24 hours with several changes of the PBS buffer. The final concentration of proteins in the so obtained dialyzed was 50mg/ml as determined by absorbance at 280 nm.

FITC Antibody Labeling

An appropriate amount of 0.5M bicarbonate buffer, pH 9.5, was added to antisera in volumetric flasks at a final antibody concentration of 20 mg/ml. According to the total amount of antibody to be tagged, 0.01 mg fluorescein per milligram of protein was added to the bicarbonate buffer equivalent to 1/10 (w:w) of IgG. The FITC solution was added slowly to the antibody solution, keeping it continuously at 4 °C for 18 hours and avoiding the foam formation. Afterward, the unbound FITC was removed by dialysis against PBS for 48 hours followed by elution with 0.01M PBS (pH7.2) through a DEAE52 column. The FITC-conjugated antibody was then divided into aliquots, lyophilized and stored at -20 °C till further use.

Identification of Working Concentrations for the FITC-Conjugated Antibody

The FITC-conjugated antibody was serially diluted in PBS (1:2, v:v) so as to obtain several sequential halving concentrations. These antibody dilutions were then used to stain frozen sections from inguinal lymph nodes of pigs infected with S. suis 2 so as to establish a maximum effective dilution. The maximum effective dilution was defined as the maximum possible antibody dilution that would specifically detect the antigen. Throughout these studies, FITC-conjugated antibody dilutions that were 2-4 times higher than the maximum effective dilution were used to reliably detect S. suis 2.

Specificity

Inguinal lymph nodes from uninfected (control) and S. suis 2-infected young piglets were sectioned and stained with the FITC-conjugated S. suis 2 antibody and observed with a fluorescence microscope to confirm negative staining of lymph node sections from control pigs and positive staining of sections from S. suis 2-infected pigs.

Animal Inoculation

30 six-week-old SPF piglets were randomly divided into two groups and injected with either Sichuan strain ZY05719 (1x10^7 CFU/kg, 20 piglets) or aseptic medium (control, 10 piglets) through intramuscular route.

Sample Collection

Two infected piglets and one control piglet were each sacrificed at 12h, 1d, 2d, 3d, 4d, 5d, 6d, 7d, 8d, 10d after injection. All the piglets were observed for clinical symptoms before sacrifice. Samples of tonsil, lymphoid node, liver, heart, spleen, lung, and brain were collected and each tissue was divided into three portions for isolation of bacterial cultures, immunofluorescence observation and histopathologic examination, respectively. Two portions were stored at -80 °C until use, while the third portion was fixed in 10% neutral buffered formalin for histopathologic examination immediately upon sampling.

Isolation of Bacterial Cultures

Samples of tonsil, lymphoid node, liver, spleen, heart, lung, and brain from all infected and control pigs
were cultured on sheep fresh blood agar culture-medium, for 24h at 37 ºC. Parallel samples were cultured on THB fluid medium containing 2% Solcoseryl for 24h at 37 ºC 180r·m⁻¹.

Preparation of Pathological Section and Microscopic Examination

Portions of all the samples including liver, spleen, kidney, heart, lung, lymphnode, brain, tonsil, were fixed in 10% neutral buffered formalin for 24 h, and then embedded in paraffin wax. After this samples were sectioned (3-5µm) and stained with haematoxylin and eosin (HE) [8]. Then these stained sections were examined systematically under a microscope.

Staining and Microscopic Examination

Two sections from each sample were stained with the FITC-conjugated S. suis 2 antibody diluted in PBS, pH 7.2, using 1:3 and 1:6 dilutions, respectively. Sections were kept in a 37 ºC constant temperature and humidity oven for 30 minutes and then rinsed slowly with PBS. Sections were then air dried and mounted on microscope spiled using carbonate glycerol and examined under a fluorescence microscope.

RESULTS

Clinical Observations of Animals

All pigs inoculated with S. suis 2 became pyrexic (39.5-41.5 ºC) for two to eight days. Most of pigs exhibited depression, anorexia. The eyes of the diseased pigs were glazed with reddening of mucous membranes (Figure 1A). Severe nervous symptoms were observed, including incoordination, lateral prostration, paddling (Figure 1B), opisthotonus, convulsions and cross-shaped of the forelimb (Figure 1C). Lameness was found in all 20 pigs, apparently in posterior limbs. The pigs were found dead from second day. The animals in control group were unaffected.

Specificity of Working Concentration for the FITC-Conjugated S. suis 2 Antibody

Fluorescence microscope observation of stained sections showed that uninfected tissue was negative as no specific fluorescence was detected in sections of lymph nodes incubated with the FITC-S. suis 2 antibody (Figure 2A,C,E,G,I,K,M). On the other hand, bright green/yellow specific fluorescence was observed in sections of tissues from S. suis 2-infected pigs

Figure 1: Clinical observations of infected pigs. A: The eyes of the diseased pigs were glazed with reddening of mucous membranes. B: Severe nervous symptoms were observed, including incoordination, lateral prostration, and paddling. C: The forelimb of infected pigs appeared convulsions and cross-shaped.
Localization of S. suis 2 Antigen in Tissue

The majority of immunoreactive bacteria concentrated in the crypt of the tonsil (Figure 2B), with some staining also observed in the peripheral diffuse lymphoid tissue. Substantial bacterial staining was found in the cortex and germinal center of lymph nodes (Figure 2D). In lungs, bacteria were found in the interstitial tissue (Figure 2F) and in liver, in the hepatic sinusoids (Figure 2F). Fluorescence particle in perisinusoidal space of disce could be bacteria partially phagocytosed by Kupffer's cell (Figure 2H). In spleen, bacteria were found in the vicinity of the periarterial lymphatic sheath and marginal zone, and substantial quantities of stained particles were also found in the marginal zone (Figure 2J). In kidney, most of bacteria were found in the renal corpuscles, whereas few bacteria were found in the renal tubule (Figure 2L). In the meninges, pathological damage was found in the cortex, corpus striatum, hippocampus, thalamus, hypothalamus, where bacteria may have been phagocytosed by glial cells (Figure 2N). Specificity fluorescence were not found in all negative control (Figure 2A, C, E, G, I, K, M).

Dynamic Distribution of S. suis 2 in Tissue

Isolation and Culture of Bacteria

The overall results of the detection of bacteria in different tissues from infected swine are shown in Table 1. The isolates from blood and the various tissues examined here from S. suis 2-positive piglets grew well in culture on agar supplemented with fresh sheep blood over a period of 24h, forming gray-whitish, smooth, uniform, translucent, and needlepoint size colonies of α-hemolysis. The floc on the bottom of test tube was observed 24h later, and the tissues infected with S. suis 2 were cultured on enrichment medium, revealing growth of gram-positive, chain cocobacteria.

S. suis 2 Immunofluorescence Staining

Bacteria were found in the tonsil as early as 12h after infection (Figure 3A), where. S. suis 2 could be
effectively isolated, and persisted for the remainder of the experimental period (Table 2). The number of bacteria in tonsil and intralymph node was higher than in the other tissues examined (Figure 3A, B) and started to decrease after 7 days (Table 2). Dynamic state distribution of bacteria was similar in liver, lung and kidney (Figure 3C, D, E) and slightly higher in spleen (Figure 3F). After 2 days of infection, only few bacteria could be found in these tissues, and their levels reached to the maximum level around day 3-4 following infection and then started decreasing on day 6 of post-infection until no bacteria could be detected on day 10 (Table 2). After 3 days of infection, bacteria were found in brain (Figure 3G), but later distribution of bacteria in brain was inconsistent. The number of bacteria were maximum on the 5th and 6th day after infection followed by a decrease. However, on day 10 after infection bacteria were again found in brain (Table 2). All piglets injected with vehicle were negative for S. suis 2 at all times tested.

Pathological Changes in Tissues and Organs

Microscopically, hemorrhage and congestion were observed in cardiac muscle, spleen, kidneys, liver and lungs (Figure 4A, B, C, D, E). Vacuole degeneration of myocardial (Figure 4F) and liver cells (Figure 4D), congestion of central veins (Figure 4D), vacuolization in brain tissue (Figure 4G), widened alveolar septum, narrowed alveolar space (Figure 4E), fibrin exudation in alveola (Figure 4H), the increased glomerular volume in kidney (Figure 4C) and, deformation and defluxion of epithelial cells in renal tube were seen (Figure 4C). No microscopic lesions were observed in any organ or tissue of the pigs in control groups.

DISCUSSIONS

Development of Direct Immunofluorescence for S. suis 2

Currently, PCR, multiplex-PCR, ELISA and direct isolation of bacteria are commonly used to detect the presence of infecting S. suis 2 in tissue and bodily fluids. All these methods have some disadvantages such as being time-consuming, being prone to return false positive results and inconsistency, among others. On the other hand, immunofluorescence methods have the advantage of being relatively rapid to perform and are specific as well as sensitive and accurate at the
Figure 3: immunofluorescent result of different tissue samples. A: tonsil (x40); B: lymphnodes (x40); C: liver (x40); D: lung (x40); E: kidney (x40); F: spleen (x40); G: brain (x40).
Table 2: Immunofluorescent Distribution of S. suis 2 in Different Tissues and Organs

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<thead>
<tr>
<th>Sacrifice time (after infection)</th>
<th>pig No.</th>
<th>Liver</th>
<th>Spleen</th>
<th>Lung</th>
<th>Kidney</th>
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Note: +: positive, -: negative.

Cellular level. These immunofluorescence methods can be used for detection and localization of antigen. Here, in this study, we have developed a FITC-conjugated-antibody specifically-directed against S. suis 2, and have described the conditions for direct immunofluorescence detection of the antigen in infected tissue. Indeed, our results showed that specific fluorescence could be detected in tissue of infected pigs. However, no specific fluorescence was observed in the tissue of pigs that had been injected with vehicle (controls) or in tissue previously treated with the S. suis 2 antibody. Collectively, these results indicate that our newly developed antibody can efficiently detect S. suis 2 in infected tissue.

Localization of S. suis 2 Antigen and Pathological Changes in Tissues and Organs

Young piglets are usually infected with S. suis 2 via airborne transmission. Once bacteria enter the bloodstream, they rapidly interact with epithelial cells, enter different tissues, and cause tissue damage. The first step of an efficient infection is the breaking through epithelial mucosae of the upper respiratory tract from where bacteria diffuse throughout the body via the general circulation. On the other hand, the bacteria located in the tonsil can enter the general circulation directly through the tonsil vascular system. However, because of the limited number of afferent lymphatic vessel of palatine tonsil, S. suis 2 usually enters the general circulation through the tonsil located in the oral cavity. Our present data are consistent with the results reported by William and Salles [9,10] who found that S. suis 2 was located in the tonsillar crypts of palatine tonsil and through crypts of palatine tonsil, the bacteria disseminated to the blood. However, some S. suis 2 may also be phagocytosed by macrophages in the tonsillar crypts of palatine tonsil. We found S. suis 2 localized in the marginal zone of spleen. This is consistent with the notion that the marginal zone of the spleen is an important site for antigen acquisition, antigen recognition and immune response. Compared to kidney, there were more S. suis 2 in the liver and spleen. S. suis 2 was mainly localized in the renal glomerulus, probably reflecting bacterial entry through the renal vasculature. Sparse bacteria were also detected in other tissues, such as red pulp of the spleen. The mechanism of induction of meningitis by S. suis 2 is currently unknown. Meningitis induced by S. suis 2 affected mainly in the cortex, and accordingly S. suis 2 were detected in this area.
Figure 4: Pathological changes in tissues and organs. A: Hemorrhage and congestion were observed in cardiac muscle. H.E 400×. B: Hemorrhage and congestion were observed in spleen. H.E 400×. C: Hemorrhage and congestion were observed in kidney, the increased glomerular volume in kidneys, deformation and defluxion of epithelial cells in renal tube were seen. H.E 400×. D: Congestion of central veins in liver. H.E 400×. E: Widened alveolar septum, narrowed alveolar space, congestion were seen in lungs. H.E 400×. F: Vacuole degeneration of myocardial cells. H.E 400×. G: Vacuolization in brain tissue. H.E 400×. H: Fibrin exudation in alveola. H.E 400×.
The number of S. suis 2 antigen located in different tissues and organs were dissimilar, and the extent of pathological changes in tissues and organs were determined by the number of S. suis 2 in them. Consistent results of this study may provide further knowledge for the future research about the pathogenicity of S. suis 2.

Dynamic Distribution of S. suis 2 and Pathological Changes in Tissue and Organs

S. suis 2 were persistent in the palatine tonsils of the piglets infected either naturally or experimentally, and were detectable as early as 12 hours of infection. S. suis 2 were also detected gradually in heart, liver, spleen, kidneys and lungs of young piglets for the first days after infection. The pathological changes in these tissues and organs were different because of the time and the number of S. suis 2. These results will hopefully provide fundation for future studies involving the understanding of infection mechanisms and pathogenesis of S. suis 2.

REFERENCES


