



NOTE

Pathology

Septicemic *Actinobacillus suis* infection in a neonatal piglet with multifocal necrotic glossitis

Kennosuke SUGIE¹⁾, Tetsuya KOMATSU¹⁾, Eri WATANDO¹⁾, Nanami INABA¹⁾, Kiyoko KATO²⁾, Daisuke TAKAMATSU^{3,4)}, Hiroya ITO³⁾ and Tomoyuki SHIBAHARA^{5,6)*}

¹⁾Aichi Prefectural Chuo Livestock Hygiene Service Center, 1-306 Jizono, Miaicho, Okazaki, Aichi 444-0805, Japan

²⁾Tochigi Prefectural Central Livestock Health and Hygiene Center, 6-8 Hiraide-kogyodanchi, Utsunomiya, Tochigi 321-0905, Japan

³⁾Division of Bacterial and Parasitic Disease, National Institute of Animal Health, National Agriculture and Food Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

⁴⁾The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1193, Japan

⁵⁾Division of Pathology and Pathophysiology, National Institute of Animal Health, National Agriculture and Food Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

⁶⁾Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58 Rinku-oraikita, Izumisano, Osaka 598-8531, Japan

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ABSTRACT. Five-day-old neonatal piglets presented with debilitation and anastasia. At the necropsy of one piglet, the apex of the tongue was found to be discolored dark red, and disseminated white foci were found on the cut surface. Many white foci were also found in the lungs and on the serosa of the liver and spleen. Histopathological findings revealed multifocal necrotic glossitis and pneumonia with Gram-negative bacilli. The bacilli were identified as *Actinobacillus suis* through immunohistochemical, biochemical, and genetic tests, including 16S rRNA gene sequencing. Although *A. suis* usually causes inflammation in thoracic and abdominal organs, lesions were also found in the tongue in the present case. This study is the first report of glossitis caused by *A. suis*.

KEY WORDS: *Actinobacillus suis*, glossitis, neonatal piglet, pneumonia

Members of the genus *Actinobacillus* are facultative anaerobic, nonmotile, Gram-negative bacilli [10]. Among *Actinobacillus* spp., *A. suis* is an important pathogen of swine which can cause disease in pigs of all ages [1, 13]. There is a difference in the tendency of clinical signs between young and adult pigs. While fattening pigs, gilts, and sows present dermatitis and abortion [9, 11, 16, 23], sudden death and increased mortality rate are observed among suckling and weaned pigs [18, 22]. However, there is no information on neonatal piglets affected with *A. suis*.

A. suis resides in the tonsils and upper respiratory tract of the soft palate of swine, where it persists harmlessly in many cases [6]. The bacilli cause diseases when stress factors, including weaning, parturition, and transportation, are applied on a farm under good sanitation [12, 13].

A. suis can cause a variety of conditions, including enteritis, mastitis, metritis, abortion, meningitis, arthritis, and sepsis [8, 15]. Histopathologically, vascular embolized thrombus and necrosis have been observed in the liver, spleen, kidneys, heart, lungs, lymph nodes, intestine, skin, central nervous system, and joints [11, 16, 18, 22, 23]. However, lesions in the tongue have not been reported to date.

Outbreaks of *A. suis* infection have been reported in the United States, Canada, Australia, and Croatia [9, 22]. However, there is only one case report in Japan, which had described sepsis with fibrinous pleuropneumonia in a weaned piglet [18].

In the current study, we performed postmortem examination of a neonatal piglet and diagnosed the case as an *A. suis* infection with multifocal necrosis in the tongue. In addition, we immunohistochemically analyzed the distribution of the bacterium in the body, inferred the developmental mechanism of glossitis, and compared the findings with those of the previous report [18].

In a farrow-to-finish farm in Aichi prefecture where 200 pigs are raised, a litter of 5-day-old neonatal piglets showed debilitation

*Correspondence to: Shibahara, T.: tshiba@affrc.go.jp

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and anastasia in February 2018. The farrowing house on the farm was kept clean, and no antibiotics were administered to the piglets. Cross-fostering had been practiced to create litters of equal sizes and reduce competition among the litters [3]. The sows were vaccinated against swine erysipelas, Japanese encephalitis, porcine parvovirus infection, porcine reproductive and respiratory syndrome (PRRS), porcine epidemic diarrhea (PED), transmissible gastroenteritis, atrophic rhinitis, and porcine circovirus type 2 (PCV2). The sows in the farrowing house were fed with antibiotics, including tylosin, amoxicillin, and sulfamonomethoxine.

One of the piglets was euthanized and subjected to necropsy. At necropsy, the apex of the tongue was found to be discolored dark red (Fig. 1a), and multifocal disseminated white foci were seen in its cross sections (Fig. 1b). Numerous multifocal white foci were also found in the lungs, and their surroundings had a red appearance due to hyperemia or hemostasis. Several white foci were also found on the serosa of the liver and spleen but not in the parenchyma. The mesenteric lymph nodes were enlarged, and the stomach was empty. No other lesions were found in any other organ.

Tissue samples of the tongue, lungs, heart, kidneys, liver, spleen, stomach, intestines (duodenum, jejunum, ileum, cecum, and colon), tonsils, trachea, lymph nodes (inguinal and mesenteric), pancreas, and central nervous system (cerebrum, middle brain, cerebellum, pons, medulla oblongata, and spinal cord) were fixed in 10% neutral phosphate-buffered formalin. The fixed samples were then embedded in paraffin wax, sectioned (approximately 3- μ m thick sections), and stained using hematoxylin-eosin and Gram's method for histopathological examination.

Histopathologically, multifocal necrosis was observed in the tongue (Fig. 1c) and lungs. Gram-negative bacilli and large numbers of denatured neutrophils with nuclear elongation (oat-like cells) were observed in the lesions (Fig. 1d and 1e). They were surrounded by macrophages and fibrocytes. Glossal ulceration was also observed. While bulging focal necrotic lesions with oat-like cells and macrophages were found only in the serous membranes of the liver (Fig. 1f) and spleen, a few focal necrotic lesions were also found in the parenchyma. In the central nervous system, the meninges were infiltrated with neutrophils. The ventricles (the lateral, third, and fourth ventricles and the aqueduct of the midbrain) and the central canal of the spinal cord were diffusely dilated from being filled with neutrophils and bacilli. Furthermore, areas adjacent to the affected ventricles exhibited extensive inflammation, with neutrophils and focal necrosis. Slight necrotic lesions were also observed in the heart and mesenteric lymph nodes.

All formalin-fixed paraffin-embedded (FFPE) tissue samples, which were cut into 3- μ m-thick sections, were subjected to immunohistochemical examination for detection of *A. suis* antigens. The primary antibody was an anti-*A. suis* rabbit antibody [18], which was used in a 1:2,048 dilution with a commercially available antibody diluent (S3022, Dako North America Inc., Carpinteria, CA, U.S.A.). The procedure was performed in accordance with the instructions in a commercial kit (Histofine Simple Steiner MAX-PO (MULTI) kit, Nichirei, Tokyo, Japan). Negative controls were obtained by using normal goat serum as the primary antibody.

Numerous positive reactions against anti-*A. suis* antibodies were observed in the FFPE samples of the tongue (Fig. 1g) and lungs. Moreover, moderate positive reactions were observed in the necrotic lesions of the central nervous system and the serous membranes of the liver and spleen. A few positive reactions were observed in the FFPE samples of the heart and mesenteric lymph nodes.

In order to amplify the 16S rRNA gene of the bacteria present in the tissue sections, DNA was extracted from the FFPE tissue sections of the tongue and lungs using a commercially available extraction kit (QIAamp DNA FFPE Tissue Kit, QIAGEN Inc., Venlo, Germany) as instructed by the supplier. Subsequently, a part of the 16S rRNA gene was amplified from the extracted DNA by performing PCR with primers 522F (5'-AAGGGACGGCTAACTACGTGCCA-3') and 1104R (5'-TTGCGGGACTTAACCCAACATCT-3') using the KOD FX polymerase (TOYOBO Co., Ltd., Osaka, Japan). The amplified fragments were sequenced by the same primers using the BigDye terminator V3.1 cycle sequencing kit and a 3130xl Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The sequences were assembled and analyzed using SEQUENCHER Ver. 5.4.1 (Gene Codes Corp., Ann Arbor, MI, U.S.A.) and EzBioCloud (<https://www.ezbiocloud.net/>) [24], respectively.

The results of EzBioCloud analysis of the 437-base-pair (bp) (tongue) and 557-bp (lungs) nucleotide sequences determined in this study revealed that DNA fragments amplified from the FFPE tissue sections possessed nucleotide sequences identical to those of the type strain of *A. suis* (DDBJ/EMBL/Genbank accession No. CP009159).

For bacterial isolation, tissue homogenates prepared from the main organs, including the liver, spleen, kidneys, heart, lungs, and cerebrum, were spread onto sheep blood agar, deoxycholate-hydrogen sulfide-lactose agar, and chocolate agar (Nissui Pharmaceutical Co., Tokyo, Japan) plates. These plates were incubated at 37°C in the presence of 5% carbon dioxide under aerobic or anaerobic conditions for 18 hr. Consequently, Gram-negative bacilli were isolated from the liver, spleen, lungs, and cerebrum on sheep blood agar and chocolate agar media.

The bacilli were beta-hemolytic, catalase-positive, and oxidase-positive. Using a commercially available biochemical identification kit (HN-20-Rapid, Nissui Pharmaceutical Co.), the seven-digit biochemical profile number of an isolate (strain D34726) from the lungs was determined to be 7117573, which yielded an over 99% relative probability of the isolate being *A. suis*. In the biochemical identification kit, the result for acid production from mannitol was negative, which is only seen in *A. suis* and not in other species belonging to genus *Actinobacillus*.

The nucleotide sequence of the 16S rRNA gene of strain D34726 was determined as described elsewhere [5]. Furthermore, a DNA homology search was performed using EzBioCloud as described above. The nucleotide sequence of the 16S rRNA gene of strain D34726 (1268 bp) was identical to that of the *A. suis* type strain ATCC 33415 (accession no. CP00915), followed by the type strains of *Actinobacillus equuli* subsp. *equuli* (NCTC 8529; accession no. M75072; 99.84%) and *Actinobacillus hominis* (CCUG 19800; accession no. L06076; 98.97%). The nucleotide sequence of the 16S rRNA gene of strain D34726 has been deposited in the DDBJ/EMBL/Genbank databases under accession no. LC415151. The threshold of the 16S rRNA gene sequence similarity for separating two strains into different species has been suggested to be within 98.7–99.0% for closely related taxa [20]. Therefore, strain D34726 is likely to be *A. suis*, *A. equuli*, or *A. hominis*.

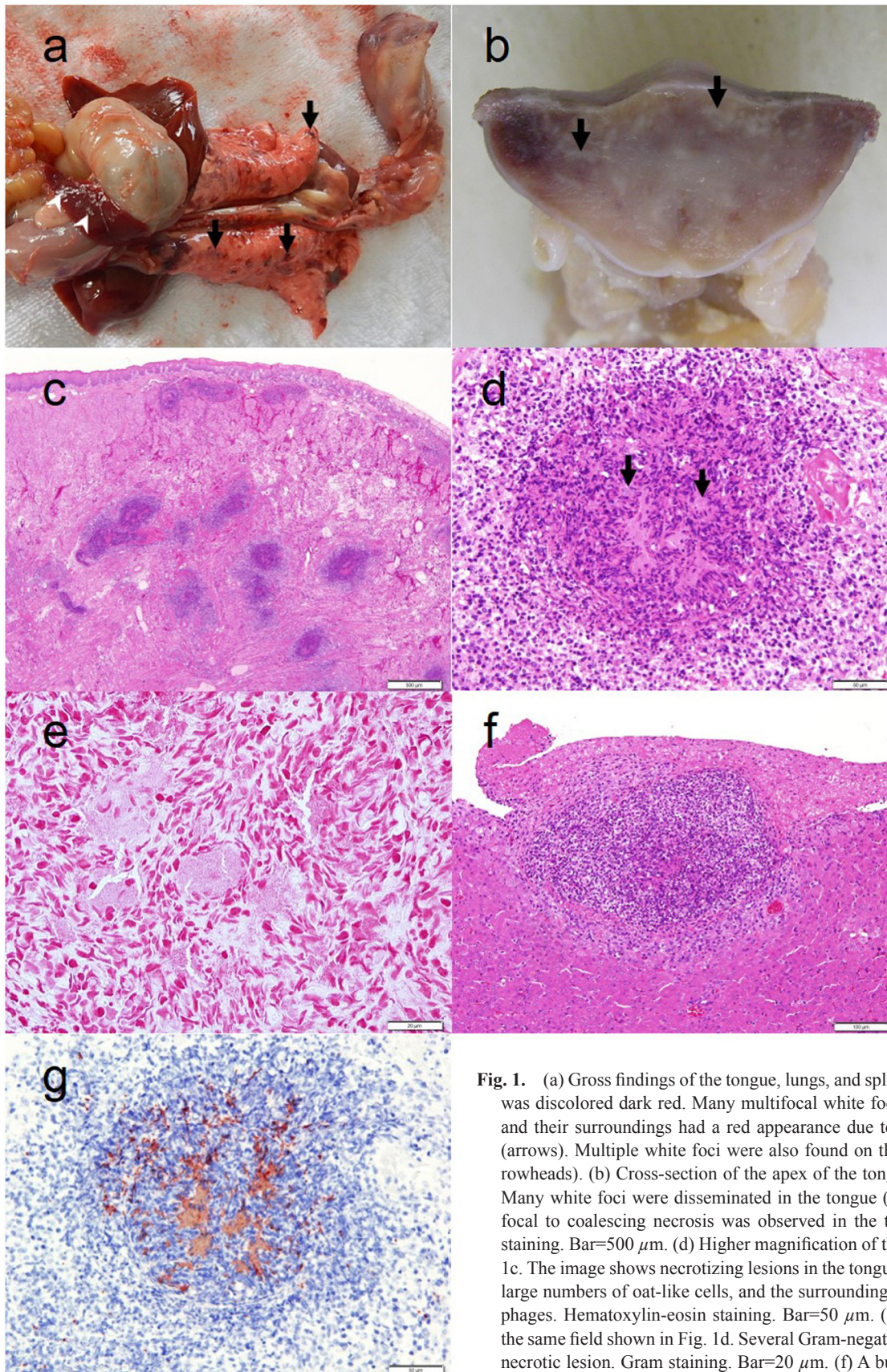


Fig. 1. (a) Gross findings of the tongue, lungs, and spleen. The apex of the tongue was discolored dark red. Many multifocal white foci were found in the lungs, and their surroundings had a red appearance due to hyperemia or hemostasis (arrows). Multiple white foci were also found on the serosa of the spleen (arrowheads). (b) Cross-section of the apex of the tongue after formalin fixation. Many white foci were disseminated in the tongue (arrows). (c) Diffuse multifocal to coalescing necrosis was observed in the tongue. Hematoxylin-eosin staining. Bar=500 µm. (d) Higher magnification of the same field shown in Fig. 1c. The image shows necrotizing lesions in the tongue, with bacilli (arrows) and large numbers of oat-like cells, and the surrounding are infiltrated with macrophages. Hematoxylin-eosin staining. Bar=50 µm. (e) Higher magnification of the same field shown in Fig. 1d. Several Gram-negative bacilli are located in the necrotic lesion. Gram staining. Bar=20 µm. (f) A bulging focal necrotic lesion in the serous membrane of the liver. Hematoxylin-eosin staining. Bar=100 µm. (g) The same field shown in Fig. 1d, but subjected to anti-*Actinobacillus suis* immunohistochemical analysis. The bacilli in the necrotic lesions of the tongue showed a positive reaction. Bar=50 µm.

Table 1. Results of antimicrobial susceptibility testing and zone diameter interpretive standards

Antimicrobial disk	Disc content ($\mu\text{g}/\text{disc}$)	Zone diameter (mm)			Diameter of inhibition circle of strain D34726	Susceptibility of strain D34726
		Resistant (R)	Intermediate (I)	Susceptible (S)		
Ampicillin	10	–	–	≥ 27	17.1	R or I
Amoxicillin	25	≤ 14	15–20	≥ 21	19.4	I
Ceftiofur	30	≤ 17	18–20	≥ 21	17.0	R
Oxytetracycline	30	≤ 14	15–18	≥ 19	17.2	I
Florfenicol	30	≤ 18	19–21	≥ 22	19.6	I
Chloramphenicol	30	–	–	≥ 28	22.5	R or I
Sulfamethoxazole–trimethoprim	25	–	–	≥ 24	18.8	R or I
Enrofloxacin	5	≤ 16	17–20	≥ 21	19.7	I

The breaking points of these antibiotics were determined on the basis of the zone diameter interpretive standards of *Pasteurella multocida* for the VKB discs and *Pasteurella* spp. for the Sensi-Disks in the package insert.

Since it is difficult to distinguish between closely related species solely by comparison of 16S rRNA gene sequences, we performed PCR toxin gene profiling of *Actinobacillus pleuropneumoniae* RTX toxins (ApxI, ApxII, ApxIII, and ApxIV) as described elsewhere [4, 19]. The results of PCR toxin gene profiling were positive for *apxICA*, *apxIICA*, and *apxIBD*, but negative for *apxIIICA* and *apxIIIBD*, which matches the profile observed in *A. suis* and *A. pleuropneumoniae* serovars 1, 5, 9, 11, and 16, but not in *A. equuli* or *A. hominis*. In addition, strain D34726 was negative for *apxIV*, which is specific to *A. pleuropneumoniae*. These results, in conjunction with the biological and biochemical characteristics described above, indicated that strain D34726 was indeed *A. suis*.

To examine the antimicrobial susceptibility of strain D34726, a disk diffusion test was performed in accordance with the Clinical and Laboratory Standard Institute method [2]. The antibiotic disks used in this study included the BD BBL Sensi-Disks (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.) for ampicillin, amoxicillin, oxytetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim and the VKB disks (Eiken Chemical Co., Tokyo, Japan) for ceftiofur, florfenicol, and enrofloxacin. Because the zone diameter interpretive standards for *A. suis* are not available, the breaking points of these antibiotics were determined on the basis of the zone diameter interpretive standards of *Pasteurella multocida* for the VKB discs and *Pasteurella* spp. for the Sensi-Disks in the package insert.

Strain D34726 was resistant to ceftiofur and exhibited intermediate susceptibility to amoxicillin, oxytetracycline, florfenicol, and enrofloxacin. The strain was also resistant or intermediately susceptible (not susceptible) to ampicillin, chloramphenicol, and sulfamethoxazole-trimethoprim (Table 1).

For virologic testing, PCR analysis was performed for PRRS virus, PCV2, classical swine fever virus, PED virus, and Getah virus, and no specific gene of any of these viruses was detected.

Our results in this study confirmed a diagnosis of septicemic *A. suis* infection. The most interesting finding of this case was the presence of multifocal necrotic glossitis associated with *A. suis* infection. *A. suis* infection is usually seen as vascular embolized thrombus as well as necrosis in thoracic and abdominal organs, skin, and the central nervous system [10, 11, 18, 22, 23]. The present case is the first to demonstrate necrosis in the tongue caused by *A. suis* infection.

In the tongue, the apex was discolored dark red. Histologically, ulceration was observed, and some of the necrotic foci exhibited angiocentric features. These findings present the following three possibilities for where the causative pathogen was transported from: (1) The tongue was injured by an external factor, such as inappropriate tooth clipping, following which the bacteria directly invaded the tongue; (2) the bacteria were hematogenously transferred from the tonsils or upper respiratory tract, where *A. suis* is usually present [6]; or (3) the glossitis might have been lesions secondary to pneumonia. It was not possible to delineate which of the three was the cause in this study.

In addition to the tongue, necrotic foci were also found in various other organs. In the liver and spleen, inflammation was observed mainly in the serosa. In the central nervous system, lesions were located along the path of the cerebrospinal fluid. Immunohistochemically, the tongue and lungs exhibited a significant positive reaction against anti-*A. suis* antibodies. On the basis of these findings, we speculate that the bacilli were hematogenously transported to each organ of the body through the cerebrospinal fluid and peritoneum.

In many previous reports, researchers have diagnosed *A. suis* infection on the basis of the biochemical characteristics and *apx* genotype profile of the isolated bacteria [9, 22]; only one report to date has described the diagnosis of *A. suis* infection by 16S rRNA gene sequencing [18]. In the current study, the identity of *A. suis* was confirmed by biochemical characterization, *apx* genotype profiling, and 16S rRNA sequence analysis, which can help specifically detect authentic *A. suis*. Thus, our study is the first to definitively demonstrate the association of authentic *A. suis* with swine glossitis.

Strain D34726 exhibited resistance or intermediate susceptibility to all of the evaluated antimicrobials. Since no antibiotics had been administered to the piglets, strain D34726 might have been originally carried by the sows and transferred to the piglets. Although the sows were fed with tylosin, amoxicillin, and sulfamonomethoxine, strain D34726 showed resistance or intermediate susceptibility to ampicillin, ceftiofur, oxytetracycline, florfenicol, chloramphenicol, sulfamethoxazole-trimethoprim, and enrofloxacin. It should especially be noted that strain D34726 showed resistance to ceftiofur, a third-generation cephalosporin,

and intermediate susceptibility to enrofloxacin, a fluoroquinolone. Deliberate use of these antibiotics is generally recommended as the final resort for antimicrobial treatment. We should be watchful for the development of antibiotic resistance in *A. suis* as well as other microorganisms isolated from pigs in the farm.

A. suis is an opportunistic infectious agent and has been reported to occur in swine herds with high health status [12]. The farrowing house in the present farm was cleanly maintained, and the sows were administered vaccines and antibiotics. Although cross-fostering improves the survival of piglets in the pre-weaning term [21], frequent mixing remains stressful for both piglets and sows [14]. In addition, stress and immunosuppression induce opportunistic infections [12, 13]. Following the present outbreak, the farmer fed adequate colostrum to piglets immediately after delivery, and subsequent occurrence was not confirmed. Colostrum provides newborn piglets with immunoglobulin G for passive immunity, which is essential for health and survival [7, 17]. These facts suggest that the affected piglets in the present case were not sufficiently immunized and might have developed infection because of the stress caused by cross-fostering.

In conclusion, this study reports the first case of glossitis due to *A. suis* infection in a piglet. Since an untypical pathology of *A. suis* infection has only been observed in this case to date, further investigation is needed to understand the pathogenicity of *A. suis* isolates by careful clinical and pathological examination in field cases as well as by infection experiments.

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