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NOTE

Bacteriology

Pathogenic characterization of *Erysipelothrix rhusiopathiae* Met-203 type SpaA strains from chronic and subacute swine erysipelas in Japan

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ABSTRACT. To characterize the *Erysipelothrix rhusiopathiae* Met-203 type surface protective antigen (Spa) A strains causing swine erysipelas in Japan, the nucleotide sequence of the hypervariable region of the *spaA* gene was determined in 80 *E. rhusiopathiae* (serotype 1a) isolates collected from pigs with chronic and subacute swine erysipelas in 14 prefectures in 2008–2014. In this study, 14 (17.5%) isolates were Met-203 type SpaA strains. We confirmed the pathogenicity of a Met-203 type SpaA strain in specific-pathogen-free pigs. In this experiment, the two challenged pigs displayed arthritis, urticaria and other clinical signs, but recovered within 10 days. Our results reveal the existence of the *E. rhusiopathiae* Met-203 type strains that have been causing chronic erysipelas in Japan.

KEY WORDS: *Erysipelothrix rhusiopathiae*, pathogenicity, pig, *spaA*

Erysipelothrix rhusiopathiae is a Gram-positive bacillus and causative agent of swine erysipelas (SE). The clinical signs of SE can be divided into three types: acute (septicemia), subacute (urticaria) and chronic (arthritis, lymphadenitis and endocarditis) [20]. Surface protective antigen (Spa) A is well known as one of the major protective antigens of *E. rhusiopathiae* [7, 21]. The N-terminal half of the hypervariable region of SpaA in particular has been shown to be important for specific immunity to the infection [3, 13, 17]. On the other hand, Harada *et al*. [2] demonstrated that SpaA was responsible for the adhesion of *E. rhusiopathiae* to porcine endothelial cells. Borrathbay *et al.* [1] also showed that SpaA plays a role in the pathogenesis of *E. rhusiopathiae* infection by demonstrating that the 50% lethal dose (LD_{50}) of the Δ*spaA* mutant was lower than that of the parental strain in mice, when the virulence of Δ*spaA E. rhusiopathiae* strain was examined.

Recently, septicemic erysipelas infection caused by a Met-203 type strain of *E. rhusiopathiae*, in which the amino acid at position 203 of the hypervariable region of SpaA is methionine, was reported by Kanda *et al*. [5]. To *et al.* [18] showed that most of the *E. rhusiopathiae* isolates from the 2008–2011 outbreaks were serotype 1a of the Met-203 type strain and suggested that these types of isolates might be widespread in Japan. We also reported that *E. rhusiopathiae* Met-203 type strains with serotype 1a of septicemic form were isolated on farms in Japan from 2008 to 2010 [19]. Although many *E. rhusiopathiae* Met-203 type strains from acute SE cases have been reported, a recent study reported on the isolation of some *E. rhusiopathiae* Met-203 type strains from subacute or chronic SE cases and their acriflavine resistance patterns and pathogenicity profiles in mice [18]. Meanwhile, Zou *et al.* [22] reported that a methionine at amino acid position 203 of the hypervariable region of the *spaA* gene is not directly related to the virulence of *E. rhusiopathiae*.

According to the Slaughterhouse Act of Japan (Act No. 114 of August 1, 1953), a carcass with a lesion from *E. rhusiopathiae* infection must be condemned. Therefore, the economic losses caused by chronic SE are of great importance to the pig industry. According to Japanese national statistics, the number of condemned pigs with SE in Japanese slaughterhouses has increased since 2009, with sharp increases in 2012 and 2013 [8, 9].

With the aim of contributing information about the recent situation concerning chronic SE and the characteristics of *E. rhusiopathiae* Met-203 type strains, we determined the nucleotide sequence of the hypervariable region of the *spaA* gene of *E. rhusiopathiae* (serotype 1a) isolated from chronic and subacute SE, and investigated their pathogenicities in mice and pigs.

A total of 80 *E. rhusiopathiae* isolates were used in this study (Table 1). They were isolated from pigs with chronic or subacute

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Table 1. Origin and classification by year of 80 *E. rhusiopathiae* strains (serotype 1a) isolated in 14 prefectures

Origin	No. of isolates classified by year							Total
	2008	2009	2010	2011	2012	2013	2014	
Urticaria	1	1			1			3
Endocarditis	3	1			7	6		17
Arthritis	7	11	10	11	10	6	5	60
Total	11	13	10	11	18	12	5	80

Table 2. SpaA types and the number of isolates collected by year

Strain or SpaA type [a)]	Difference of nucleotide [b)] (amino acid position [b,c)])			No. of isolates by year							Total (%)
	584 (195)	609 (203)	726 (242)	2008	2009	2010	2011	2012	2013	2014	
Ile-203	GAT(Asp)	ATT(Ile)	GAG(Glu)	5	12	8	9	10	7	5	56 (70.0)
Ala-195	G<u>C</u>T(<u>Ala</u>)	ATT(Ile)	GAG(Glu)	6	1						7 (8.8)
Met-203	GAT(Asp)	AT<u>G</u>(<u>Met</u>)	GAG(Glu)			2		7	5		14 (17.5)
Met-203/Asp-242	GAT(Asp)	AT<u>G</u>(<u>Met</u>)	GA<u>T</u>(<u>Asp</u>)				2	1			3 (3.7)

a) SpaA types were determined according to sequence of the 432-bp hypervariable region of the *spaA* gene. b) Underlined letters: nucleotides that differ from Ile-203 type strain at the same position in the protein sequence. Underlined amino acid abbreviations: amino acids that differ from Ile-203 type strain at the same position in the protein sequence. c) Asp:Aspartic Acid, Ala:Alanine, Ile:Isoleucine, Met:Methionine, Glu:Glutamic Acid.

erysipelas from 14 Japanese prefectures (Iwate, Miyagi, Akita, Fukushima, Ibaraki, Gunma, Saitama, Chiba, Kanagawa, Niigata, Fukui, Mie, Kyoto and Kumamoto) from 2008 to 2014 and were collected from Livestock Hygiene Service Centers and official meat hygiene inspections. From them, sixty isolates originated from arthritis cases, 17 from endocarditis cases and 3 from urticaria cases. *E. rhusiopathiae* Marienfelde strain (serotype 1a) was used for the growth agglutination (GA) tests.

E. rhusiopathiae was grown in tryptose phosphate broth and agar (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 0.1% Tween 80 (pH 7.6). To isolate *E. rhusiopathiae* from the experimentally infected animals, the selective medium or agar was supplemented with kanamycin (500 μg/m*l*) and gentamicin (25 μg/m*l*) [16].

A 432-bp fragment of the N-terminal half of the *spaA* gene was PCR amplified and sequenced directly, using previously described methods [19]. The DNA data were analyzed with Genetyx Network ver. 9.0.4 (Genetyx Corp., Tokyo, Japan). Based on the sequence analysis, 80 field isolates were classified into four types (Table 2). Fifty-six of the isolates (70%) had isoleucine at amino acid position 203 (Ile-203), which were the predominant type and constantly observed in 2008–2014. This sequence was reported in the *E. rhusiopathiae* strains of various serotype, such as 1a, 2 (GeneBank accession number AB259654), 8 (AB259656) and 17 (AB259660) [17] and also in the *E. rhusiopathiae* Koganei 65–0.15 live vaccine strain (AB024082) [4]. We confirmed that 52 of 56 Ile-203 strains were amplified by SNP-based PCR assay for detection of the *E. rhusiopathiae* live vaccine strain [14], which included just slight amplification. Fourteen isolates (17.5%) were Met-203-type strains, which were observed in 2010, 2012 and 2013, and were often isolated next to the Ile-203 type in these years. Three isolates (3.7%) had methionine and aspartic acid at amino acid positions 203 and 242, respectively (Met-203/Asp-242), which were also seen around the same period (2011–2012). In contrast, the Ala-195 type strain was only seen in 2008–2009.

The pathogenicity tests in mice and pigs were performed using the modified methods of Takahashi *et al.* [15]. All the animal experiments were conducted in accordance with the National Veterinary Assay Laboratory Guide for the Care and Use of Laboratory Animals and the Animal Welfare Acts of Japan. We used 4-week-old female ddY mice (Nippon SLC, Hamamatsu, Japan) and 3-month-old specific-pathogen-free (SPF) pigs (Zen-Noh Live Stock Co., Ltd., Tokyo, Japan). The LD_{50} value in mice of each of 10 *E. rhusiopathiae* isolates (6 from endocarditis cases and 4 from arthritis cases) was determined using the method of Kärber [6]. In mice, the LD_{50} values (log CFU/mouse) of seven Met-203 type strains (endocarditis origin: 5 and arthritis origin: 2) were 0.30–1.33, while those of the three Met-203/Asp-242 type strains (endocarditis origin: 1and arthritis origin: 2) were 0.81–1.24.

We selected the Met-203 type strain (Met-203/Fukui) from arthritis SE as the challenge strain for pigs, because it had the lowest LD_{50} value in mice. Two pigs were challenged intradermally in the flank with 0.1 m*l* of the challenge strain (7.1×10^7 CFU/pig). Another pig was kept with the challenged pigs in the same pen as a control. Clinical signs in the pigs were observed for 14 days after challenge. Feces from the rectum were collected from day 1 to day 10 after challenge. Serum samples were collected at the point of challenge and at autopsy (14 days after challenge). To determine the anti-erysipelas antibody titers, a GA test with the *E. rhusiopathiae* Marienfelde strain was performed as described previously [12].

The results of a challenge study in pigs are shown in Table 3. After challenge with the Met-203/Fukui strain (LD_{50}: 0.77 logCFU/mouse), the two challenged pigs showed the following clinical signs: depression, anorexia, dysstasia, pyrexia (42.1°C and 41.7°C), lameness from arthritis and systemic urticarial lesions. The urticarial lesions were observed as purplish red regions (maximum size of erythema: 3.5 cm ×3.0 cm and 4.0 cm ×3.5 cm) of anthema at injection site and appeared on the 2nd day after challenge. The urticarial lesions in the pigs were observable for about a week and then gradually disappeared. Lameness from arthritis was observed on the 7th day after challenge, followed by recovery. The clinical signs in the two pigs had disappeared

Table 3. Clinical responses and bacterial shedding from pigs after challenge with the *E. rhusiopathiae* Met-203/Fukui strain

Group	Pig No.	Clinical responses[a)]			Growth agglutinating antibody titer		Bacterial shedding in the feces[b)]	Re-isolation of challenge strain from organs[c)]
		Pyrexia (°C)	Erythema	Arthritis	Before challenge	14 days after challenge		
Challenge	P-12	42.1	systemic	+	<4	256	+	iliac lymphonodi, tonsils, synovial fluid
	P-13	41.7	systemic	+	<4	128	+	iliac lymphonodi, tonsils, synovial fluid
Control	P-14	39.5	-	-	<4	<4	-	-

a) Challenge strain (7.1×10^7 CFU/pig) was not lethal to pigs, and all the clinical responses to it disappeared within 10 days. -; no response. b) +: The challenge strain was recovered from feces on days 2 to 4 after challenge exposure. c) Organs tested:liver, spleen, kidney, heart, lung, iliac lymphonodi, tonsils and synovial fluid.

completely 10 days after challenge. The control pig showed no clinical signs of infection. The two challenged pigs discharged the bacterium in their feces on days 2–4 post-challenge; the bacterium was the same serotype and had the same *spaA* gene fragment as that of the challenge strain.

The serum samples collected before the challenge from the three pigs had a GA antibody titer of less than 4. At day 14 post-challenge, the GA antibody titers of the two challenged pigs were 256–128 each, but that of the control pig remained less than 4 (Table 3).

Fourteen days after challenge, all three pigs were euthanized and autopsied. Samples for bacterial isolation were collected from the main organs (liver, spleen, kidney, heart, lung and iliac lymphonodi) and from the tonsils and synovial fluid. Isolation and serotyping of the bacteria from each sample were performed by the method of Takahashi *et al*. [16], and the sequence of the *spaA* gene fragment of the isolated bacteria was confirmed. At autopsy, the two challenged pigs showed no lesions, but a small increase in slightly cloudy serosanguineous synovial fluid was noted. The control pig showed no signs of disease. The serotype and *spaA* sequences of 432-bp hypervariable region of the strains isolated from the iliac lymphonodi, tonsils and synovial fluid of the two challenged pigs were the same as those of the challenge strain. *E. rhusiopathiae* was not isolated from the main organs of the control pig (Table 3).

In this study, we investigated *E. rhusiopathiae* Met-203 strains in the context of chronic or subacute SE, because it often causes whole carcasses to be condemned in slaughterhouses. We showed that 14 of 80 isolates (17.5%) were Met-203 type strains, and 3 isolates (3.7%) were Met-203/Asp-242 type strains. Both the Met-203 type and Met-203/Asp-242 type strains were observed in 2010–2013, and these were often isolated next to Ile-203 type strains in these years. Because the acute form of the Met-203 type strain became prevalent after 2008 [5], we surmise that infections caused by the chronic form of this strain may have occurred after numerous outbreaks of the acute form on farms. This could be one of the factors responsible for the recent increase in the number of condemned pigs in slaughterhouses. In addition, we think that the Ile-203 type strains amplified by SNP-based PCR [14] need further investigation about their association with the *E. rhusiopathiae* Koganei 65–0.15 live vaccine strain.

When Nagai *et al.* [10] reported the sequence of the 432-bp hypervariable region of the *spaA* gene as one of the new strain-typing methods for discrimination of the live vaccine strain, *E. rhusiopathiae* Met-203 type strains were not isolated among the 16 *E. rhusiopathiae* strains (serotype 1a: 10 and serotype 1b: 6) isolated in 2001–2002 from arthritis cases in the Chubu area of central Japan. However, To *et al*. characterized 83 *E. rhusiopathiae* strains from eight Japanese prefectures in 2008–2011 and showed that *E. rhusiopathiae* Met-203 type strains were isolated from acute, subacute and chronic forms [18]. Our sequence analysis is chronologically consistent with these previous reports [10, 18].

The LD_{50} values (0.30–1.33) of the Met-203 type strains and Met-203/Asp-242 type strains from chronic SE origin reflect their very high virulence in mice. These LD_{50} values are comparable with those of our previous report of Met-203 type strains from acute SE cases [19]. Our findings also support the results of other studies in that the majority of strains that caused chronic infections were highly virulent in mice [4, 10, 11].

We examined the pathogenicity of the Met-203/Fukui strain in SPF pigs with negative GA titers and examined whether the bacteria were shed and transmitted by contact. In the challenged pigs, two had arthritis, urticaria and other clinical signs, but recovered within 10 days. The pathogenicity of the Met-203/Fukui strain was obviously lower than that of the Met-203 type strain of septicemia origin reported in our previous study, which was lethal in pigs [19]. However, after recovery, the challenged strain was isolated from the iliac lymphonodi, tonsils and synovial fluid of the challenged pigs; therefore, these pigs were potential carriers of this pathogen. Some temporary bacterial shedding was also observed in the feces of both challenged pigs, but the control pig showed no clinical signs and its GA antibody titer remained less than 4. In our clean experimental environment, contact infection was not observed; however, on farms, *E. rhusiopathiae* is probably spread by oral infection from contact with infected feces.

E. rhusiopathiae Met-203 type strains are considered to be highly pathogenic and cause acute SE characterized by sudden death in pigs [5, 19]. On the other hand, *E. rhusiopathiae* Met-203 type strains have been isolated from chronic SE. The factor of chronic SE depends not only on the virulence of the *E. rhusiopathiae* strain but also on the immune status of the pig. And, the variations in the pathogenicity of the Met-203 type strains are still unclear. We have shown that one of the *E. rhusiopathiae* Met-203 type strains caused arthritis SE and that chronic SE causing *E. rhusiopathiae* Met-203 type strains exist to a certain extent in Japan.

Therefore, it is important to continue monitoring *E. rhusiopathiae* field isolates and to confirm the prevalence and pathogenicity of the field strains by sequencing the hypervariable region of the *spaA* gene and with pathogenicity tests.

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