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NOTE

Bacteriology

Single nucleotide polymorphism genotyping of *Erysipelothrix rhusiopathiae* isolates from pigs affected with chronic erysipelas in Japan

Kazumasa SHIRAIWA[1)], Yohsuke OGAWA[1)], Sayaka NISHIKAWA[1)], Masahiro KUSUMOTO[1)], Masahiro EGUCHI[1)] and Yoshihiro SHIMOJI[1,2)]*

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

[2)]Research Institute for Biomedical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

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ABSTRACT. Over the past decades, *Erysipelothrix rhusiopathiae* strains displaying similar phenotypic and genetic profiles of the attenuated, acriflavine-resistant *E. rhusiopathiae* Koganei 65-0.15 strain (serovar 1a) have been frequently isolated from pigs affected with chronic erysipelas in Japan. In this study, using the conventional PCR assay that was designed to detect strain-specific single nucleotide polymorphism (SNP) sites found in the genome of the vaccine strain, we analyzed *E. rhusiopathiae* isolates from pigs with chronic disease in farms where the Koganei vaccine was used. Out of a total of 155 isolates, 101 isolates (65.2%) were determined to be the vaccine strain by SNP-based PCR. Among the 101 PCR-positive isolates, four isolates were found to be sensitive to acriflavine.

KEY WORDS: *Erysipelothrix rhusiopathiae,* Koganei 65-0.15 strain, SNP-based PCR, strain-specific detection, vaccine

Swine erysipelas is a disease caused by the gram-positive facultative intracellular pathogen *Erysipelothrix rhusiopathiae* [9]. The disease may present as acute septicemia, subacute urticaria, or chronic endocarditis and polyarthritis, all of which result in great economic losses to the pork industry worldwide [9]. In Japan, an attenuated live vaccine (Koganei 65-0.15 strain, serovar 1a) (abbreviated as Koganei) has long been used to control the disease. The vaccine strain was attenuated by 65 passages on agar plates containing 0.15% acriflavine dye [7], showing that acriflavine-resistance is a marker of the vaccine strain. However, some field isolates also display acriflavine-resistance [1, 2, 5, 6], indicating that this phenotype is not a suitable marker for the strain. Differentiation of the vaccine strain from field isolates has been performed by a random amplified polymorphic DNA (RAPD) analysis [1]. In that study, field isolates belonging to a specific RAPD type, namely RAPD 1-2, were determined to be Koganei strains.

Recently, we developed a single nucleotide polymorphism (SNP)-based conventional PCR assay that can detect Koganei-specific SNP sites in the genome and can differentiate Koganei strains from field isolates displaying similar phenotypic and genetic profiles to the vaccine strain [8]. In this system, after comparing the draft genome sequence of the Koganei strain (GenBank accession no. DRA003556) with the reference whole genome sequence of the Fujisawa strain (accession no. AP012027) [3], a total of 76 SNPs were identified, and five SNP sites were selected for PCR-detection using primers that were designed to amplify DNA fragments including the SNP sites from the Koganei genome [8]. In this study, to gain insight into the epidemiology of chronic erysipelas and to investigate the possible association of the live Koganei vaccine strain with disease, we tested the SNP-based PCR assay for *E. rhusiopathiae* strains isolated from pigs with chronic diseases.

E. rhusiopathiae strains were isolated at prefectural livestock hygiene centers or meat inspection centers in 10 prefectures located either in Tohoku, Kanto, Hokuriku, Tokai, Chubu, Chugoku, Shikoku or Kyushu region of Japan during 2014 and 2016 from pigs affected with erysipelas. They were sent to the National Institute of Animal Health for serological and genetic analyses. In this study, only the *E. rhusiopathiae* isolates from pigs affected with chronic erysipelas, namely, endocarditis and polyarthritis, in farms where the Koganei vaccine were used were analyzed for their serovars and acriflavine-resistance, and tested by the SNP-

*Correspondence to: Shimoji, Y., National Institute of Animal Health, NARO, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan.
e-mail: shimoji@affrc.go.jp

Table 1. Genotyping results with the SNP-based PCR of *E. rhusiopathiae* clinical isolates from pigs with chronic erysipelas

Serovars identified	No. of isolates per serovar	No. of PCR-positive isolates using the following primer sets[a)]					No. of acriflavine-resistant isolates among the PCR-positive strains[b)]
		0001F & 0001R	0543F & 0543R	0636F & 0636R	1398F & 1398R	1449F & 1449R	
1a	103	101	101	101	101	101	97
1b	8	0	0	0	0	0	-[c)]
2	44	0	0	0	0	0	-

a) The sequences of the primers were reported in Shiraiwa *et al.* [8], b) Isolates resistant to 0.0025 to 0.005% were defined as acriflavine resistant, c) Not applicable.

based PCR [8].

The *E. rhusiopathiae* strains were grown at 37°C for 16 hr in brain heart infusion broth (Becton, Dickinson and Company., Baltimore, MD, U.S.A.) supplemented with 0.1% Tween 80 and 0.3% Tris-HCl (pH 8.0) (BHI-T80). The serovars of the strains were determined by a double agar-gel precipitation test as previously described [1]. Acriflavine resistance was tested as previously described [1]. In short, bacterial strains were streaked on BHI-T80 agar plates containing 0.02, 0.01, 0.005, 0.0025 or 0% acriflavine (Wako Pure Chemical Industries Ltd., Osaka, Japan). The plates were incubated in a 10% CO_2 incubator at 37°C for 4 days, and the bacterial growth was observed. For the PCR assay, the genomic DNA of the *E. rhusiopathiae* strains was prepared using an alkaline boiling method as previously described [4]. Briefly, a single colony of each strain was suspended in 50 μl of 25 mM NaOH and incubated at 95°C for 5 min. After neutralization of the suspension with the addition of 4 μl of 1 M Tris-HCl (pH 8.0), the suspension was centrifuged (15,000 × g), and 5 μl of the supernatant was used for PCR (total 50 μl). PCR amplification was performed using KOD FX DNA polymerase (TOYOBO, Osaka, Japan) and a BIO-RAD T-100 thermal cycler (BIO-RAD, Hercules, CA, U.S.A.) with the following conditions: initial denaturation, 95°C for 5 min; and 3 amplification steps with 35 cycles of 95°C for 30 sec, 67°C for 30 sec and 72°C for 30 sec as previously described [8].

In this study, it was found that 103, 8 and 44 isolates belonged to serovars 1a, 1b and 2, respectively, among the 155 *E. rhusiopathiae* isolates tested (Table 1). All the isolates were tested with the SNP-based PCR using the five sets of primers. We confirmed that 101 isolates out of the 103 serovar 1a isolates were 100% positive for all the five primer sets and were thus determined to be Koganei strain. We confirmed that the serovar 1a strains were either 100% positive or 100% negative for the five primer sets, and we did not observe any positive reactions with the other serovar strains. Among the 101 PCR-positive isolates, 97 isolates showed resistance to acriflavine, whereas four were sensitive.

Thus, our results showed that 65.2% of 155 field isolates from pigs affected with chronic erysipelas in farms where the Koganei vaccine were used were Koganei strain. This high rate of detecting the vaccine strain is not surprising as Imada *et al.* [1] reported that 266 of the 524 (50.8%) strains of serovars 1a, 1b and 2, all of which are dominant serovars in clinical isolates [9], belonged to the RAPD 1-2 type and therefore were determined to be Koganei strain. Very importantly, among the PCR-positive strains, four PCR-positive isolates were found to be sensitive to acriflavine. The loss of acriflavine-resistance in the RAPD 1-2 type strains has been shown [1], thus clearly indicating that acriflavine resistance is not an appropriate marker of the vaccine strain.

We confirmed that the SNPs used in the PCR system were not observed in the whole genome sequences of 34 wild-type serovar 1a strains isolated in the Japanese pig population during the past two decades (data not shown), thus suggesting that these SNPs used for PCR identification are Koganei-specific and might have been generated during the agar attenuation process using acriflavine dye. The attenuation mechanisms of the vaccine are unknown, and therefore, we assume that our approach based on whole genome sequence data is reasonable and the best method of identifying the Koganei strain.

Finally, this SNP-based PCR assay provided rapid detection of the Koganei vaccine strain, and it will help in facilitating early diagnosis of swine erysipelas in meat inspection centers and prefectural livestock hygiene centers.

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