# Isolation of Acriflavine Resistant *Erysipelothrix rhusiopathiae* from Slaughter Pigs in Japan

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ABSTRACT. *Erysipelothrix rhusiopathiae* is the causative agent of swine erysipelas. Although an attenuated vaccine is used in Japan, recent increases in disease occurrence have cast doubts on its efficacy. We investigated the similarity between the vaccine strain and *E. rhusiopathiae* field isolates by the analysis of acriflavine resistance (the vaccine strain marker), serotype, DNA fingerprinting and pathogenicity to mice. Although 7 acriflavine resistant *E. rhusiopathiae* isolates were separated from arthritic lesions of slaughter pigs, we were unable to prove that they were identical to the vaccine strain. — KEY WORDS: acriflavine, *Erysipelothrix rhusiopathiae*, vaccine.
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*Erysipelothrix rhusiopathiae* is the causative agent of economically important swine erysipelas, and also an important pathogen in the field of public health as it is the cause of erysipeloid in humans [8]. In Japan, to protect against swine erysipelas, an acriflavine fast attenuated strain Koganei 65–0.15 (serovar la; abbreviated as Kg-la), obtained from a virulent strain by 65 passages on agar media containing 0.15% acriflavine dyes, is used as a live vaccine for piglets [3, 4]. From around 1972, the occurrence of swine erysipelas decreased greatly with the increase in the amount of the vaccine produced. However, after about 1985, although piglets are generally vaccinated annually, the occurrence of chronic endocarditis and polyarthritis of swine has continued to increase. A possible reason for this increase may be interference with vaccine immunogenicity by the presence of maternal antibody [9].

In this paper, we investigated the similarity between the vaccine strain and *E. rhusiopathiae* isolates from slaughter pigs by the analysis of acriflavine resistance, serotype, DNA fingerprinting, and pathogenicity to mice.

First, 50 $\mu l$ of $1 \times 10^4$ to $10^5$ cells of reference *Erysipelothrix* strains used for serotyping [1, 5, 6] were inoculated on Brain Heart Infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% Tween-80 (BHIT) and acriflavine (Kanto, Co., Ltd., Japan). Acriflavine was gradually diluted two-fold (range 0.2–0.005% at the final concentration) in order to measure minimum inhibitory concentration (MIC). After incubation at 37°C for 24 hr, resistant strains uniformly could grow but sensitive strains were not able to grow on the acriflavine containing agar plates. The vaccine strain Kg-la could grow on the agar plates containing acriflavine at a concentration of 0.1%, but the reference strains were not able to grow at 0.005% (Table 1), reconfirming that acriflavine resistance was an useful marker for distinguishing the vaccine strain from non-vaccine strains. Next, the acriflavine resistance of 27 *E. rhusiopathiae* field isolates collected from the arthritic lesions of 27 slaughter pigs which had been vaccinated with Kg-la, was examined (Table 2). All strains were isolated in 4 slaughter houses in 1995 from 27 different breeding farms, where the vaccination by Kg-la had apparently been performed. Twenty-one of the 27 field isolates were serotype 1a, 5 were serotype 2, and only 1 was serotype 1b. A total of 7 isolates were resistant to acriflavine; all of them were serovar 1a (Table 2). Although, Kg-la could grow at a concentration of more than 0.12%, none of the field isolates could (Table 2). When $1 \times 10^9$ cells of the acriflavine resistant isolates and Kg-la were subcutaneously injected into mice, arthritis appeared in all mice. All mice challenged by the acriflavine resistant field isolates died within 7 days, while mice challenged by the vaccine stain survived (Table 2).

In order to examine the genetic relatedness among acriflavine resistant *E. rhusiopathiae* isolates and the Kg-la vaccine strain, the PCR-based DNA fingerprinting method using random amplified polymorphic DNA (RAPD) was performed. Arbitrary primers, AP46 (5'-GAGGACAAAG-3') and AP47 (5'-GCGGAAATAG-3') [2] were used. Strains genetically close to each other should show similar RAPD patterns [7].

The RAPD patterns of the 7 acriflavine resistant strains were very similar to that of Kg-la (Fig. 1, lanes 1 to 8), although there were minor differences among them. However, the RAPD patterns of acriflavine sensitive serotype 1a strain, including the reference strain ME7, were also similar to that of Kg-la (Fig. 1, all lanes except lanes 12 and 14). Since the RAPD patterns of serotype 1b and 2 were slightly different from 1a (Fig. 1, lanes 12 and 14), the RAPD data demonstrated that the serotype 1a *E. rhusiopathiae* strains were closely related genetically,

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Table 1. Acriflavine resistance among *Erysipelothrix* reference strains

| Species | Strain | Serotype | MIC of acriflavine (% w/v) |
|---|---|---|---|
| *E. rhusiopathiae* | ME-7 | 1a | 0.005 |
| | Fujisawa | 1a | 0.005 |
| | Koganei 65–0.15 | 1a | >0.12 |
| | 422/1E1 | 1b | 0.005 |
| | ATCC19414 | 2 | 0.005 |
| | Koganei2 | 2 | 0.005 |
| | Nagasaki | 2 | 0.005 |
| | R32E11 | 2 | 0.005 |
| | Tama | 2 | 0.005 |
| | Doggerscharbe | 4 | 0.005 |
| | Pécs67 | 5 | 0.005 |
| | Dolphin E-1 | 6 | 0.005 |
| | Goda | 8 | 0.005 |
| | Kaperaku | 9 | 0.005 |
| | IV12/8 | 11 | 0.005 |
| | Pécs9 | 12 | 0.005 |
| | Pécs3597 | 15 | 0.005 |
| | Tanzania | 16 | 0.005 |
| | 545 | 17 | 0.005 |
| | 2017 | 19 | 0.005 |
| | Bãno36 | 21 | 0.005 |
| | MEW22 | N | 0.005 |
| *E. tonsillarum* | ATCC43339 | 7 | 0.005 |
| | ATCC43338 | 7 | 0.005 |
| | P-43 | 7 | 0.005 |
| | L1–3 | 7 | 0.005 |
| | Witlling | 3 | 0.005 |
| | Lengyel-P | 10 | 0.005 |
| | Iszap-4 | 14 | 0.005 |
| | 2553 | 20 | 0.005 |
| | Bãno107 | 22 | 0.005 |
| | KS20A | 23 | 0.005 |
| New species 1 | Pécs56 | 13 | 0.005 |
| | Shiribeshi-17 | 13 | 0.005 |
| | Shiribeshi-19 | 13 | 0.005 |
| New species 2 | 715 | 18 | 0.005 |

* ATCC19414 and ATCC43339 were ATCC type strains for *E. rhusiopathiae* and *E. tonsillarum*, respectively. Strain Koganei is an acriflavine-fast attenuated strain and used for the vaccine production in Japan.

irrespective of their acriflavine sensitivity.

Acriflavine resistance is an useful marker for the production of the vaccine strain, but it does not always mean that acriflavine resistance is a good selection marker to discriminate the vaccines train from field isolates. In this study, we isolated acriflavine resistant *E. rhusiopathiae* isolates from the arthritis of slaughter pigs, and their pathogenicities were stronger than those of the vaccine strain, though we were unable to discriminate serotype 1a strains from each other by RAPD analysis. These results are unable to provide direct evidence that the vaccine strain is a cause of the recent increase of swine erysipelas, since we might also have separated spontaneous acriflavine resistant field isolates unrelated to the vaccine strain.

Table 2. Acriflavine resistance of *E. rhusiopathiae* field isolates

| Strain | Serotype | MIC of acriflavine (% w/v) | Challenge[a)] arthritis | Challenge[a)] death |
|---|---|---|---|---|
| Koganei 65–0.15 | 1a | >0.12 | + | – |
| S-29 | 2 | 0.005 | + | + |
| S-30 | 1a | 0.005 | NT | NT |
| S-33 | 1a | 0.005 | NT | NT |
| S-37 | 1a | 0.005 | NT | NT |
| S-38 | 1a | 0.005 | NT | NT |
| S-41 | 1a | 0.1 | + | + |
| S-42 | 1a | 0.005 | + | + |
| S-57 | 1a | 0.005 | NT | NT |
| S-58 | 1a | 0.005 | NT | NT |
| S-60 | 1a | 0.1 | + | + |
| S-61 | 1a | 0.1 | + | + |
| S-235 | 1a | 0.005 | NT | NT |
| S-236 | 1a | 0.005 | NT | NT |
| S-237 | 1a | 0.005 | NT | NT |
| S-361 | 1a | 0.08 | + | + |
| N-21 | 2 | 0.005 | NT | NT |
| N-53 | 1a | 0.1 | + | + |
| N-54 | 1a | 0.005 | + | + |
| N-59 | 1a | 0.005 | NT | NT |
| N-84 | 2 | 0.005 | NT | NT |
| H-23 | 1a | 0.1 | + | + |
| H-26 | 1b | 0.005 | NT | NT |
| H-100 | 1a | 0.005 | NT | NT |
| O-19 | 1a | 0.08 | + | + |
| O-20 | 1a | 0.005 | + | + |
| O-23 | 2 | 0.005 | NT | NT |
| O-24 | 2 | 0.005 | NT | NT |

Field isolates were derived from S, N, H, and O slaughter houses, respectively. a) Mice were subcutaneously challenged by bacterial cells of $1 \times 10^9$ CFU, and observed for 2 weeks. NT, not tested.

Recently, so-called 'high-health status' pigs, which have a low incidence of disease, are often produced in swine facilities that practice mediated early weaning programs and multiple site management in Japan. It is speculated that such pigs are more susceptible to certain diseases because of the way they are raised, and erysipelas may be one of them. The influence of vaccination on such pigs might be more severe than for normal pigs, and under such circumstances, the attenuated vaccine strains injected in the body might partially revert to the wild virulent type.

In this study, we were unable to demonstrate that the vaccine strain caused swine erysipelas, but this paper is the first report on acriflavine resistant field isolates in Japan. By further detailed epidemiological studies on *E. rhusiopathiae* field isolates, a large number of acriflavine resistant isolates would be separated and the pathogenicity of the vaccine strains would become a matter of some controversy. Consequently, since the marker of acriflavine resistance are too ambiguous, a more genetically defined vaccination system is desirable for the future.

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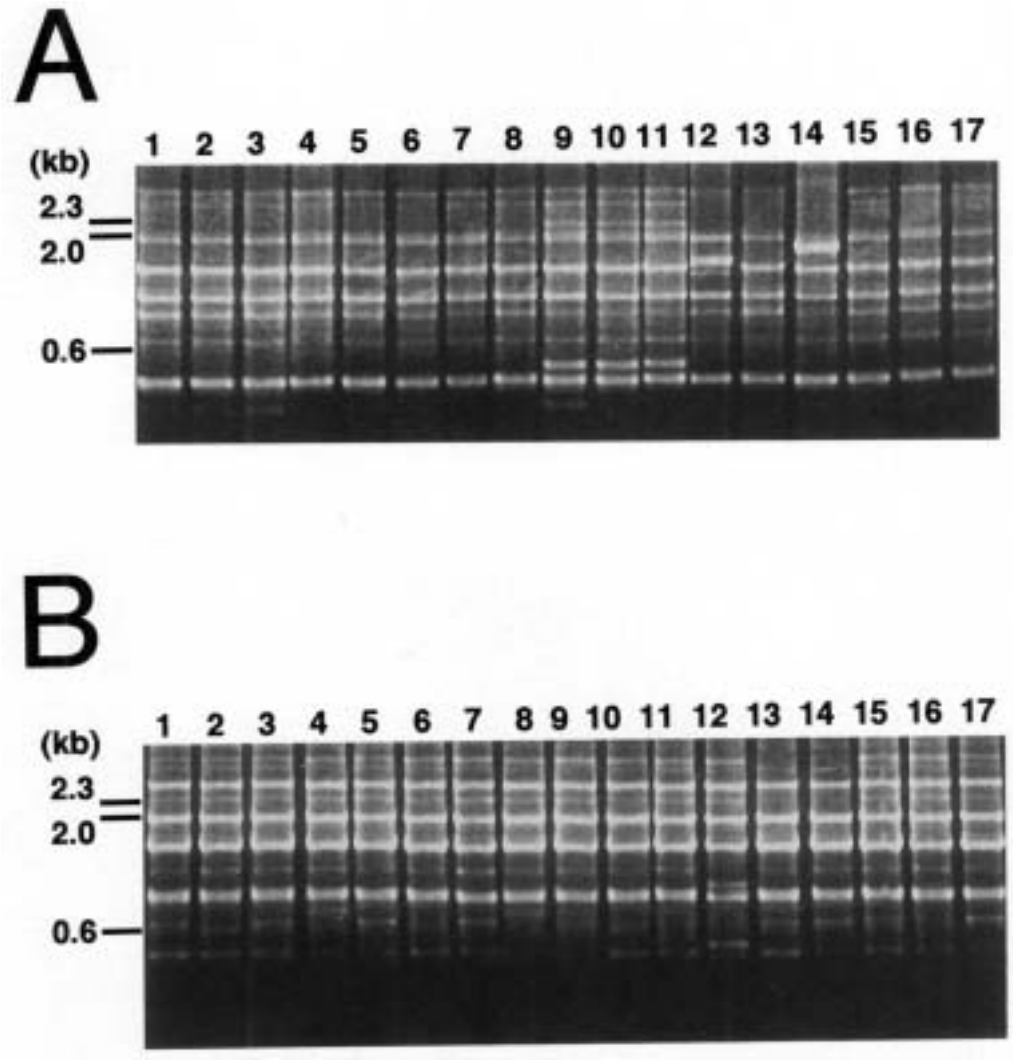


Fig. 1. Comparison of the RAPD patterns of *E. rhusiopathiae* strains. Two arbitrary primers, AP46 (A) and AP47 (B), were used. Strains used in this experiment were acriflavine resistant strains (lanes 1 to 8), and acriflavine sensitive strains (lanes 9 to 17): S-41 (lane 1), S-60 (lane 2), S-61 (lane 3), S-361 (lane 4), N-53 (lane 5), H-23 (lane 6), O-19 (lane 7), Kg-la (lane 8), S-235 (lane 9), S-236 (lane 10), S-237 (lane 11), O-23 (lane 12), N-59 (lane 13), H-26 (lane 14), H-100 (lane 15), S-58 (lane 16), ME-7 (lane 17).

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