

Etiological and Biological Characteristics of *Erysipelothrix rhusiopathiae* Isolated between 1994 and 2001 from Pigs with Swine Erysipelas in Japan

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(Received 24 July 2008/Accepted 31 December 2008)

ABSTRACT. We investigated 66 *Erysipelothrix rhusiopathiae* strains isolated from pigs affected with swine erysipelas in Japan from 1994 to 2001 for serotype, pathogenicity towards mice, protection in vaccinated mice and antimicrobial susceptibility. Most of the isolates (84.8%) were serotype 1 or 2. For the first time, strains belonging to serotype 21 were isolated from cases of septicemia. Fifty isolates (75.8%) were highly virulent, 12 isolates (18.2%) were weakly virulent and 4 isolates were avirulent strains. All the mice vaccinated with the Koganei 65-0.15 vaccine strain survived challenge exposure with 50 highly virulent isolates. Six isolates (9.1%) grew on TPB-T80 agar containing 0.02% of acriflavine, and this was identical to the growth of the vaccine strain. Forty-seven isolates (71.2%) were resistant to oxytetracycline. The number of strains resistant to oxytetracycline among field isolates increased rapidly each year. Tylosin-resistant strains were also isolated (6.1%). These results suggest that certain characteristics, particularly antimicrobial susceptibility of *E. rhusiopathiae* isolates, change yearly in the field. Therefore, further investigation of the characteristics of *E. rhusiopathiae* field isolates is necessary.

KEY WORDS: antimicrobial resistance, *Erysipelothrix rhusiopathiae*, pathogenicity, serotype, vaccine.

J. Vet. Med. Sci. 71(6): 697-702, 2009

Erysipelothrix rhusiopathiae is the causative agent of swine erysipelas, which results in great economic losses in pig industries throughout the world. The clinical signs of swine erysipelas can be classified into acute (septicemia), subacute and chronic (arthritis, endocarditis and urticaria) [23]. Approximately 2000 pigs are infected with swine erysipelas every year in Japan. *E. rhusiopathiae* is also an important pathogen with respect to public health since it causes erysipeloids in humans [23].

The swine erysipelas live vaccine prepared from the acriflavine-fast attenuated strain Koganei 65-0.15 has long been used in Japan [13]. Takahashi *et al.* [19] and Sawada *et al.* [12] reported that mice and swine immunized with the live vaccine survive challenge exposure to various serotypes of *E. rhusiopathiae* field isolates. However, there is little recent information available regarding the immunogenicity of *E. rhusiopathiae* field isolates; moreover, the effectiveness of the vaccine against *E. rhusiopathiae* strains in recent years has not been confirmed.

The vaccine strain, Koganei 65-0.15, is capable of growing in media containing 0.02% acriflavine. Therefore, the acriflavine resistance marker has been used as one of the tools for discrimination between the vaccine strain and field isolates. However, some acriflavine-resistant *E. rhusiopathiae* strains have been isolated from slaughter pigs affected by chronic arthritis, and the apparent similarity between acriflavine-resistant field isolates and the vaccine strain cannot be contradicted [3, 5, 8].

Several antimicrobial agents are used in pigs to enhance

growth and treat diseases. Tetracyclines are most commonly used for pigs in Japan [1]. Yamamoto *et al.* [24] reported that the number of antibiotic-resistant *E. rhusiopathiae* field isolates especially resistant to tetracyclines and macrolides increased from 1988 to 1998.

We investigated the etiological and biological characteristics of *E. rhusiopathiae* field isolates with the aim of contributing information for prevention of swine erysipelas.

MATERIALS AND METHODS

Bacterial strains and media: A total of 66 strains of *E. rhusiopathiae* were included in this study. They were isolated from cases of swine erysipelas between 1994 and 2001 in five prefectures (Chiba, Niigata, Toyama, Hiroshima, Yamaguchi) of Japan. Infections associated with the isolates included arthritis (50 cases), endocarditis (12 cases), urticaria (7 cases) and septicemia (4 cases). The strains were identified as *E. rhusiopathiae* on the basis of cell morphology, characteristic reactions on triple sugar iron agar slants and test-tube brush-like growth in gelatin [22]. In addition, polymerase chain reaction (PCR) for specific detection of *E. rhusiopathiae* was performed [21]. Serotyping of isolates was determined by a previously described method [18].

Lyophilized vaccines (live organisms) prepared from acriflavine-fast attenuated *E. rhusiopathiae*, strain Koganei 65-0.15 (serotype 1a), were used [13]. The reconstituted vaccine contained 1.0×10^8 viable bacteria/mL.

Animals: Four-week-old female mice of an outbred ddY strain were used. They were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan), conventionally followed up and

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Table 1. Serotypes of the 66 *E. rhusiopathiae* strains isolated from pigs from 1994 to 2001 in Japan

| Origin | No. of isolates of serotype | | | | | | | Total |
|--------------|-----------------------------|----|----|---|---|----|----|-------|
| | 1a | 1b | 2 | 6 | 8 | 19 | 21 | |
| Septicemia | 2 | | | | | | 2 | 4 |
| Urticaria | | 1 | 6 | | | | | 7 |
| Arthritis | 16 | 5 | 21 | | | | | 43 |
| Endocarditis | 1 | | 4 | 1 | 3 | 1 | | 12 |
| Total | 19 | 6 | 31 | 1 | 3 | 1 | 2 | 66 |

*: Untypable.

raised in confinement.

Pathogenicity tests: Five mice were inoculated subcutaneously in the right groin with 0.1 mL of a medium composed of tryptose phosphate (TP) broth (DIFCO) and 0.1% Tween 80 (TPB-T80) cultured for 24 hr with each isolate. Each inoculum contained approximately 10^8 viable organisms. The mice were observed every day for 10 d after exposure, and responses were determined by the quantal (live-dead) method.

Protection in vaccinated mice: Mice were inoculated subcutaneously in the right groin with 0.1 mL of erysipelas live vaccine. Five mice were inoculated subcutaneously in the left groin with 24-hr TPB-T80 cultured with each highly virulent isolate 10 d after vaccination. Each inoculum contained approximately 10^8 viable organisms. The mice were observed every day for 10 d after challenge exposure, and the mortality was recorded.

Acriflavine susceptibility testing: Each isolate was cultured in 10 mL of TPB-T80 for 24 hr at 37°C. Then, 0.1 mL of the bacterial culture of each isolate was dropped onto TPB-T80 agar containing various concentrations (0.005, 0.01, 0.02 and 0.04%) of acriflavine (Tokyo Kasei Kogyo, Tokyo, Japan) or agar that did not contain acriflavine. The agar plate was incubated for 48 hr at 37°C, and bacterial growth was examined. Acriflavine susceptibility was determined by the highest concentration at which strains showed almost the same growth as they did on the agar that did not contain acriflavine. The vaccine strain (Koganei 65-0.15) was used as a positive control strain.

RAPD typing: DNA extractions of *E. rhusiopathiae* strains were performed as previously described [6]. Randomly amplified polymorphic DNA (RAPD) typing was performed by the method described by Imada *et al.* [3]. The primer D9355 was used.

MICs: Minimum inhibitory concentrations (MICs) were determined using the standard methods of the Japanese Society of Chemotherapy for agar dilution tests in Mueller-Hinton agar (DIFCO) [4]. After inoculation, agar plates were incubated for 48 hr at 37°C. The MICs were defined as the lowest concentration of antimicrobial agents that prevented visible growth. Two-fold serial dilutions of antimicrobial agent solutions were prepared so that the concentrations of the antimicrobial agents ranged from 0.015 to 128 µg/mL. The following 17 antimicrobial agents were tested: ampicillin (ABPC), benzylpenicillin (PC-G),

ceftiofur (CTF), cefazolin (CEZ), dihydrostreptomycin (DSM), kanamycin (KM), erythromycin (EM), tylosin (TS), lincomycin (LCM), virginiamycin (VGM), doxycycline (DOXY), oxytetracycline (OTC), chloramphenicol (CP), sulfadimethoxine (SDMX), enrofloxacin (ERFX), danofloxacin (DNFX) and oxolinic acid (OXA). When the MIC distribution of antimicrobials was bimodal, the breakpoint was set at the midpoint between the peaks of each MIC distribution.

RESULTS

Serotyping isolates: The serotypes of the isolates are shown in Table 1. Most of the isolates were classified into serotype 1 (consisting of subtypes 1a and 1b) or 2. The isolates from the cases of septicemia belonged to serotype 1a or 21. Most of the isolates from the cases of urticaria were serotype 2. Isolates from the cases with arthritis were distributed almost evenly between serotypes 1 and 2. The isolates from the cases of endocarditis belonged to various serotypes.

Pathogenicity towards mice: The relationship between the origins of the *E. rhusiopathiae* isolates and their pathogenicity towards mice is shown in Table 2. All the isolates from the cases of septicemia and urticaria were highly virulent. Most of the isolates from the cases of arthritis and endocarditis were also highly virulent; however, there were some weak and non-virulent isolates. The relationship between the serotypes of *E. rhusiopathiae* isolates and their pathogenicity towards mice is shown in Table 3. Of the iso-

Table 2. Relationship between the origins of *E. rhusiopathiae* isolates from pigs with erysipelas and their pathogenicity towards mice

| Origin | No. of isolates of pathogenicity | | | Total |
|--------------|----------------------------------|-----------------|-----------------|-------|
| | H ^{a)} | W ^{b)} | N ^{c)} | |
| Septicemia | 4 | | | 4 |
| Urticaria | 7 | | | 7 |
| Arthritis | 29 | 11 | 4 | 44 |
| Endocarditis | 10 | 1 | | 11 |
| Total | 50 | 12 | 4 | 66 |

a) All mice died after subcutaneous inoculation with each strain.

b) Caused only arthritis in one or more mice.

c) Induced no clinical signs.

Table 3. Relationship between serotypes of *E. rhusiopathiae* isolates from pigs with erysipelas and their pathogenicity towards mice

| Pathogenicity | No. of strains of serotype | | | | | | | | Total |
|-----------------|----------------------------|----|----|---|---|----|----|----|-------|
| | 1a | 1b | 2 | 6 | 8 | 19 | 21 | UT | |
| H ^{a)} | 6 | 6 | 28 | 1 | 3 | 1 | 2 | 3 | 50 |
| W ^{b)} | 10 | | 2 | | | | | | 12 |
| N ^{c)} | 3 | | 1 | | | | | | 4 |
| Total | 19 | 6 | 31 | 1 | 3 | 1 | 2 | 3 | 66 |

a) All mice died after subcutaneous inoculation with each strain.

b) Caused only arthritis in one or more mice.

c) Induced no clinical signs.

Table 4. Susceptibility of the 66 *E. rhusiopathiae* strains to acriflavine

| Acriflavine susceptibility (%) | No. of strains of serotype | | | | | | | | Total # (%) |
|--------------------------------|----------------------------|----|----|---|---|----|----|----|-------------|
| | 1a | 1b | 2 | 6 | 8 | 19 | 21 | UT | |
| <0.005 | 4 | 4 | 30 | 1 | 3 | 1 | | 3 | 46 (69.7) |
| 0.005 | 3 | 2 | 1 | | | | 2 | | 8 (12.1) |
| 0.01 | 6 | | | | | | | | 6 (9.1) |
| 0.02 | 6 | | | | | | | | 6 (9.1) |
| 0.04 | | | | | | | | | 0 (0) |

Table 5. RAPD types and pathogenicity toward mice of 19 *E. rhusiopathiae* isolates of serotype 1a

| RAPD type | Acriflavine susceptibility (%) | No. of strains of pathogenicity | | | Total |
|----------------------------|--------------------------------|---------------------------------|-----------------|-----------------|-------|
| | | H ^{a)} | W ^{b)} | N ^{c)} | |
| Similar to Koganei 65–0.15 | 0.02 | 1 | 4 | 1 | 6 |
| | 0.01 | 1 | 5 | | 6 |
| | 0.005 | | | 1 | 1 |
| | <0.005 | | 1 | 1 | 2 |
| Other | 0.005 | 2 | | | 2 |
| | <0.005 | 2 | | | 2 |

a) All mice died after subcutaneous inoculation with each strain.

b) Caused only arthritis in one or more mice.

c) Induced no clinical signs.

lates belonging to serotype 1a, weak and non-pathogenic strains were more frequent than highly pathogenic strains. Most of the isolates belonging to serotypes 1b and 2 were highly pathogenic. All isolates belonging to serotypes 6, 8, 19 and 21 and all untypable (UT) isolates were highly pathogenic.

Protection studies: All the mice vaccinated with the vaccine strain survived challenge exposure to the 50 highly virulent isolates of serotypes 1a, 1b, 2, 6, 8, 19, 21 and UT.

Acriflavine susceptibility test: The results of the acriflavine susceptibility test are shown in Table 4. Of the 66 isolates, 6 (9.1%) grew on the TPB-T80 agar containing 0.02% acriflavine, which was identical to the growth of the Koganei 65–0.15 strain (positive control). All the isolates that grew on the TPB-T80 agar containing more than 0.01% acriflavine belonged to serotype 1a. On the other hand, the isolates belong to serotypes other than 1a grew on TPB-T80 agar containing 0.005% or less acriflavine.

RAPD types and pathogenicity towards mice: A total of 15 (78.9%) serotype 1a isolates showed similar RAPD patterns to that of the strain Koganei 65–0.15 (Table 5). Among the strains exhibiting similar RAPD types as Koganei 65–0.15, there were highly, weak and non-pathogenic strains. On the other hand, all isolates showing other RAPD types were highly pathogenic strains. Four isolates (21.1%) demonstrated the same characteristics as Koganei 65–0.15, i.e., they grew on agar containing 0.02% acriflavine and caused arthritis in mice.

Antimicrobial susceptibility: The MICs of the 66 isolates for the 17 antimicrobial agents are shown in Table 6. All the strains were highly susceptible to ABPC, PC-G, CTF, ERFX and DNFX (MICs 0.03–0.5 µg/mL). CEZ, VGM, OXA and CP were moderately active against all strains (MICs, 0.25–32 µg/mL). KM and SDMX showed no activity (MICs > 128 µg/mL) against any of the strains. The MICs of DSM, EM, TS, LCM, OTC and DOXY presented

Table 6. Susceptibility of the 66 isolates of *E. rhusiopathiae* to antimicrobial agents

| Antimicrobial agent | No. of isolates with MIC ($\mu\text{g/mL}$) of | | | | | | | | | | | | | | | No. of resistant strains (%) |
|---------------------|--|------|------|-------|------|-----|----|----|----|----|----|----|----|-----|------|------------------------------|
| | 0.015 | 0.03 | 0.06 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 128< | |
| Benzylpenicillin | | 2 | 39 | 7 | 17 | 1 | | | | | | | | | | |
| Ampicillin | | | 26 | 25 | 15 | | | | | | | | | | | |
| Cefazolin | | | | | 2 | 10 | 3 | 8 | 21 | 10 | 11 | 1 | | | | |
| Ceftiofur | | 1 | 14 | 3 | 47 | | | | | | | | | | | |
| Dihydrostreptomycin | | | | | | | | 3 | 17 | 13 | 10 | 7 | 1 | | 14 | 14 (21.2) |
| Kanamycin | | | | | | | | | | | | | | | 66 | |
| Oxytetracycline | | | | | | | | 12 | 7 | | 6 | 37 | 4 | | | 47 (71.2) |
| Doxycycline | | | | | | 5 | 10 | 5 | 1 | 3 | 42 | | | | | 45 (68.2) |
| Erythromycin | | | 42 | 8 | 7 | 1 | | 2 | 1 | | | | | | 4 | 4 (6.1) |
| Tylosin | | | 34 | 12 | 15 | 1 | | | | | | | | | 4 | 4 (6.1) |
| Lincomycin | | | 3 | 1 | 23 | 26 | 8 | 1 | | | | | | | 4 | 4 (6.1) |
| Virginiamycin | | | | | | 13 | 43 | 5 | 3 | 1 | 1 | | | | | |
| Sulfadimethoxine | | | | | | | | | | | | | | | 66 | |
| Oxolinic acid | | | | | | | | | | 6 | 55 | 5 | | | | |
| Enrofloxacin | | | 4 | 58 | 4 | | | | | | | | | | | |
| Danofloxacin | | | | 20 | 46 | | | | | | | | | | | |
| Chloramphenicol | | | | | | | | | | 43 | 21 | 2 | | | | |

Table 7. Relationship between resistance patterns and serotypes of the 66 *E. rhusiopathiae* isolates

| Resistance patterns ^{a)} | No. of strains of Serotype | | | | Total # (%) |
|-----------------------------------|----------------------------|---------|-----------|-------------------------------|-------------|
| | 1a | 1b | 2 | Other serotypes ^{b)} | |
| OTC, DOXY, EM, TS, LCM | 0 | 0 | 4 | 0 | 4 (6.1) |
| OTC, DOXY, DSM | 4 | 5 | 5 | 0 | 14 (21.2) |
| OTC, DOXY | 1 | 1 | 18 | 7 | 27 (40.9) |
| OTC | 2 | 0 | 0 | 0 | 2 (3.0) |
| Total # (%) | 7 (10.6) | 6 (9.1) | 27 (40.9) | 7 (10.6) | 47 (71.2) |

a) OTC: oxytetracycline. DOXY: doxycycline. EM: erythromycin. TS: tylosin. LCM: lincomycin.

b) Includes serotypes 6, 8, 21 and untypable.

two distribution peaks. The MIC breakpoints of isolates to DSM, EM, TS, LCM, OTC and DOXY in the present antimicrobial susceptibility test were 128, 128, 128, 128, 8 and 8 $\mu\text{g/mL}$, respectively. Fourteen isolates (21.2%) were resistant to DSM (MICs > 128 $\mu\text{g/mL}$), while 47 (71.2%) were resistant to OTC (MICs \geq 16 $\mu\text{g/mL}$) and DOXY (MICs \geq 8 $\mu\text{g/mL}$). Most of the isolates were susceptible to EM, TS and LCM. However, 4 isolates (6.1%) were resistant to EM (MICs > 128 $\mu\text{g/mL}$), TS (MICs > 128 $\mu\text{g/mL}$) and LCM (MICs > 128 $\mu\text{g/mL}$).

The resistance patterns for the antibiotics are shown in Table 7. Of the 66 isolates, 47 (71.2%) were resistant to one or more of the antimicrobials. Four resistance patterns were found. Isolates resistant to OTC and DOXY (40.9%) were most frequent, followed by those resistant to OTC, DOXY and DSM (21.2%); those resistant to OTC, EM, TS and LCM (6.1%); and those resistant to OTC (3.0%). All the antibiotic-resistant isolates were resistant to OTC. Of the resistant strains, 27 (57.4%) belonged to serotype 2; 7 (14.9%) belonged to serotype 1a; and 6 (12.8%) belonged to serotype 1b. All the isolates belonging to serotype 1b were resistant strains.

DISCUSSION

We demonstrated that a total of 56 (84.8%) strains belonging to the major serotypes 1a, 1b and 2, and 10 (15.2%) strains belonging to serotypes 6, 8, 19, 21 and UT were isolated. This is in general agreement with the results for *E. rhusiopathiae* strains isolated from 1983 to 1993 [15] and 1995 to 2004 [7], indicating that serotypes 1 and 2 have remained dominant in Japan. It should be noted that 2 strains belonging to serotype 21 were isolated from cases of septicemia in the present investigation. This is the first report of isolation of serotype 21 strains from pigs affected with acute septicemic erysipelas in Japan. In a previous study [15], only strains of serotypes 1a and 2 were isolated from cases of septicemia. These serotype 21 strains were isolated from non-vaccinated pigs from the same farm. Challenge exposure by intradermal inoculation in the flanks of pigs with broth culture of these serotype 21 strains caused a general urticarial lesion with profound depression and anorexia (data not shown). This observation is found rarely in pigs that undergo challenge exposure with strains belonging to serotypes other than 1 and 2.

The pathogenicity of isolates for mice classified according to origin and serotype was in accordance with previous

results [17]. No correlation between pathogenicity and serotype was observed. Recently, molecular biology methods, such as randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE), have been used to differentiate *Erysipelothrix* species and strains [9, 10]. These methods might also be useful in epidemiological studies. Further investigations are needed to clarify the taxonomic relationship between the serotypes and pathogenicity of the genus *Erysipelothrix*.

Data available concerning survival rates in vaccinated mice indicates that protection against challenge exposure with various serotypes of *E. rhusiopathiae* occurs in mice. Based on our results, it is likely that the immunity induced by the vaccine is still effective in mice. However, protection in vaccinated mice cannot always be correlated with that in vaccinated swine [12, 19].

Makino *et al.* [5] investigated the similarity between the vaccine strain and *E. rhusiopathiae* field isolates by analysis of acriflavine resistance, serotype, RAPD and pathogenicity towards mice. They separated acriflavine-resistant field isolates from slaughter pigs but were unable to demonstrate a correlation between acriflavine-resistant field isolates and the vaccine strain. Imada *et al.* [3] insist that several serotype 1a field isolates are derived from the live vaccine strain based on the results of RAPD typing. In the present study, we also reported acriflavine-resistant field isolates showing the same characteristics as Koganei 65–0.15. Sawada *et al.* [11] and Nitta *et al.* [8] reported that PFGE is a useful tool for discriminating the live vaccine strain from field isolates. Further molecular biological and epidemiological studies on the relationship between acriflavine-resistant field isolates and the vaccine strain are needed; moreover, investigation of more useful markers for the vaccine strain should be sought for more adequate quality control and quality assurance of the live vaccine.

The results for the susceptibility of *E. rhusiopathiae* strains to ABPC, PC-G, CP, KM and SDMX were in general agreement with those reported previously [24]. PC-G and ABPC had high activities against *E. rhusiopathiae* [7, 20]. This indicates that PC-G and ABPC are antibiotics of choice for treatment of swine erysipelas. *E. rhusiopathiae* was also highly susceptible to CTF and fluoroquinolones as described previously [24]. In Japan, EM and DSM have been used for treatment of porcine bacterial respiratory diseases. Takahashi *et al.* [16] reported that the frequencies of resistance to DSM and EM are 17.4 and 5.8%, respectively. The frequency of resistance to DSM increased in the period between 1988 and 1998 (37.0%) [24], but decreased from 1994 to 2001 (20.6%). The frequency of resistance to EM decreased in 1988–1998 (1.9%) [24], but increased during 1994–2001 (6.1%). No increasing or decreasing tendency was recognized in the frequency of resistance to either antibiotic; however, resistant strains do exist [7, 20].

Although TS has been used for more than 30 years as a feed additive and therapeutic agent, TS-resistant *E. rhusiopathiae* strains were detected in the strains isolated in 2001. Takahashi *et al.* [20] reported that 13% of *E. rhusiopathiae*

strains isolated in an abattoir from 2003 to 2005 were TS-resistant. The reason for the emergence and increase in TS-resistant *E. rhusiopathiae* strains is unclear, but this may be correlated with cross-resistance to other macrolides, such as EM and tilmicosin.

The number of strains resistant to OTC among field isolates has increased rapidly each year. OTC has long been administered to pigs as a feed additive to promote growth and as a veterinary treatment. In general, the use of antimicrobial agents in animal feeds for both veterinary treatment and growth promotion has increased the presence of resistant bacteria in animals [2, 14]. Yamamoto *et al.* [25] demonstrated the presence of *tet(M)* in field isolates of tetracycline-resistant *E. rhusiopathiae* and suggested that tetracycline-resistant *E. rhusiopathiae* strains acquire the *tet(M)* gene from the *Enterococcus*, *Streptococcus* and *Listeria* species in digestive tracts harbouring conjugative plasmids or transposons carrying the *tet(M)* gene. The mechanism of transmission of the *tet(M)* gene and the manner in which this gene has spread to *E. rhusiopathiae* strains have yet to be clarified.

Our results show that the etiological properties of *E. rhusiopathiae* have not significantly altered. However, we isolated acriflavine-resistant *E. rhusiopathiae* strains that were similar to the vaccine strain. It is very important to clarify the relationship between acriflavine-resistant field isolates and the vaccine strain. In addition, the number of antibiotic-resistant strains among field isolates has increased. Moreover, Miyao *et al.* reported the presence of fluoroquinolone-resistant *E. rhusiopathiae* field isolates in Japan for the first time [7]. Therefore, continued monitoring of swine erysipelas, collection of *E. rhusiopathiae* field isolates and further investigation of the characteristics of *E. rhusiopathiae* remains necessary.

ACKNOWLEDGEMENT. We are grateful to the staff of the Livestock Hygiene Service Centers across the country for providing us with *E. rhusiopathiae* strains.

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