Multiplex PCR assay for the simultaneous detection and differentiation of clonal lineages of *Erysipelothrix rhusiopathiae* serovar 1a strains currently circulating in Japan

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**ABSTRACT.** The species *Erysipelothrix rhusiopathiae* displays genetic heterogeneity; however, *E. rhusiopathiae* serovar 1a strains currently circulating in Japan exhibit remarkably low levels of genetic diversity and group into clonal sublineages of Lineage IVb (IVb-1 and IVb-2). In the present study, based on whole genome sequencing data, we designed primers for a multiplex PCR assay to simultaneously detect and differentiate the sublineages of *E. rhusiopathiae* strains. Among the one hundred and twenty-seven isolates of various serovar strains, including isolates from a wide range of hosts and geographic origins, the PCR assay could successfully detect and differentiate the serovar 1a strains belonging to the sublineages.

**KEY WORDS:** clonal Lineage IVb, *Erysipelothrix rhusiopathiae*, multiplex PCR

*Erysipelothrix rhusiopathiae* is a gram-positive intracellular pathogen that is ubiquitous in nature and causes a variety of diseases called erysipelas in many animals, including human and birds [18]. In swine, this organism can cause acute septicemia, subacute urticaria, or chronic endocarditis and polyarthritis, all of which result in great economic losses to the swine industry worldwide [18].

Serovars of *E. rhusiopathiae*, which are determined with a double agar-gel precipitation test using type-specific rabbit antisera and heat stable peptidoglycan antigens, are closely related to the clinical forms [18]; among the serovars assigned to the species *E. rhusiopathiae* (serovars 1a, 1b, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19, 21, 23 and N, which lacks serovar-specific antigens), serovars 1a and 2 are predominant in acute and chronic diseases, respectively [1, 5, 8, 9, 12–14, 17, 18].

Recently, whole genome sequence data obtained from *E. rhusiopathiae* isolates from a wide range of hosts and geographic origins revealed the species *E. rhusiopathiae* is comprised of three distinct clades (Clades 1, 2 and 3), which are not clearly segregated by serovars, host species or geographic origins, and an “intermediate” clade between Clade 2 and the dominant Clade 3 [2]. Whole genome analysis of a large collection of *E. rhusiopathiae* isolates from arctic and boreal ungulate populations in North America also confirmed the genetic heterogeneity of the isolates [3]. By contrast, genome-wide single nucleotide polymorphism (SNP) analysis of serovar 1a isolates collected after 2007 from acute/subacute swine erysipelas from various regions in Japan showed that these isolates exhibit remarkably low levels of genetic diversity and group into either of two clonal lineages (designated Lineage IVb-1 and IVb-2) within the intermediate group [7]. Furthermore, these isolates, which may have stemmed from an ancestor common to Chinese strains, independently evolved and spread separately, at least in Kyushu and Honshu islands [7]. In Japan, the incidence of acute swine erysipelas due to *E. rhusiopathiae* serovar 1a has been increasing since 2008 [7, 14–16]. Taken together, these findings suggest that the clonal lineage strains belonging to the intermediate group might be an emerging clone in eastern Asian countries; however, it remains unknown why these strains emerged in these areas and whether other factors, including other environmental and/or host factors, are involved in the outbreaks [7].

In the present study, to further investigate the epidemiology of the recent increased number of cases of acute swine erysipelas in Japan, we developed an inexpensive and quick alternative method to whole genome sequencing for the identification of the *E.*
rhusiopathiae clonal strains currently circulating in Japan. Using primers designed based on genome-wide SNP data, we developed a multiplex PCR technique that enables the simultaneous detection and differentiation of the clonal lineages of *E. rhusiopathiae*.

*E. rhusiopathiae* strains were grown at 37°C for 16 hr in brain heart infusion broth (Becton, Dickinson and Co., Baltimore, MD, U.S.A.) supplemented with 0.1% Tween 80 and 0.3% Tris-HCl (pH 8.0). The serovars were determined using a double agar-gel precipitation test as previously described [5]. Based on the genome-wide SNP data among the *E. rhusiopathiae* strains collected over a period of two decades [7], we selected SNP sites common and/or specific for clonal lineages, Lineages IVb-1 and IVb-2.

According to the previously described method [11], the primers were designed to form a mismatch at the base adjacent to the SNP site in the forward primers; SNPs were located in the 2nd position, and an artificial mismatch base was introduced in the 3rd position from the 3′ end of the forward primers. The synthetic oligonucleotide primers used were 0022F3 (5′- GGATGTTATATTCTCGCTCAAGGGCCA-3′) and 0022R3 (5′- CTGTTTTGAGTTCATTCACTTCATCCA-3′) for the detection of Lineage IVb-1 and IVb-2 strains, 0533F (5′-AATGACTATCGCAAAGAGCAAGGAAAAACTG-3′) and 0533R (5′-ATCATCTTGCGTCACCGCAACACGACGTGA-3′) for the detection of Lineage IVb-1 strains, and 0655F2 (5′-TAAATCATGTAGCATTAACGTCTCAGC-3′) and 0655R1 (5′-CCATCTTTACCGATGTATAAGGGTACA-3′) for the detection of Lineage IVb-2 strains. PCR was performed using a BIO-RAD T100 thermal cycler (BIO-RAD, CA, U.S.A.), and the specificity of the primers was empirically assessed after changing the lengths of the primers and optimizing annealing temperatures. The PCR conditions were finally determined as follows: initial denaturation at 95°C for 5 min; and three steps of amplification (35 cycles) at 95°C for 30 sec, 67°C for 30 sec, and 72°C for 40 sec with a reaction mixture (25 µl) containing 25 ng of template DNA, 0.3 µM of each primer for the six primers, 0.4 mM each of dNTP, PCR buffer, and 0.5 U of KOD FX DNA polymerase (TOYOBO, Osaka, Japan).

The multiplex PCR assay comprised three sets of primers: the Lineage IVb-specific primer set targeting an SNP in the DNA mismatch repair protein gene *mutL* (ERH 0022), and the sublineage-specific primer sets, each targeting an SNP in the single-stranded DNA-specific exonuclease gene *recJ* (ERH 0533), and the UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase gene *murF* (ERH 0655) for detection of Lineages IVb-1 and IVb-2, respectively. The PCR with primers 0022F3 and 0022R3 amplified a 1,195-bp fragment from all the Lineage IVb strains tested, and the PCR with primer sets 0533F and 0533R and 0655F2 and 0655R1 amplified 851- and 574-bp fragments from Lineages IVb-1 and IVb-2, respectively (Fig. 1).

The specificity of the SNP-based PCR assay was examined using Japanese strains of Lineages I, II, III and IV, all of which were serovar 1a strains isolated over the last two decades [7], other various serovar strains from pigs affected with acute and/or chronic diseases and other strains with different host origins, including *Erysipelothrix tonsillarum* strains. The PCR assay simultaneously detected and differentiated 21 strains belonging to the clonal IVb-1 and IVb-2 sublineages, and there were no positive reactions with other lineage strains and other serovar strains, demonstrating that the multiplex PCR showed 100% specificity with the strains tested (Tables 1 and 2).

*E. rhusiopathiae* expresses SpaA (Surface Protective Antigen A), a choline-binding protein anchored to the phosphorylcholine on the cell surface [4, 10]. The hypervariable region in *spaA* has been used for single-locus sequence-based genotyping of *E. rhusiopathiae* [6, 14–16, 19]. We recently reported that the phylogenetic classification results obtained from the genome-wide SNP analysis of *E. rhusiopathiae* Japanese serovar 1a isolates were consistent with the results obtained from the *spaA* genotyping [7], indicating that *spaA* genotyping may become a practical alternative to whole genome sequencing of Japanese isolates. In that study, it was revealed that increased cases of acute swine erysipelas after 2007 in Japan have been exclusively caused by two clonal lineage strains (Lineage IVb-1 and IVb-2) with a specific *spaA* genotype, namely M203/I257-SpaA, in which the amino acid residues at positions 203 and 257 are methionine and isoleucine, respectively. However, among the 34 field isolates analyzed,
### Table 1. Multiplex PCR results with *E. rhusiopathiae* serovar 1a strains from different lineages

| Lineage | No. of strains tested | Multiplex PCR results with primers | Remarks |
|---------|------------------------|------------------------------------|---------|
| I       | 7                      | 0022F3 & 0022R3 0533F & 0533R 0655F2 & 0655R1 | I203/L257-SpaA type |
| II      | 6                      | - - - | I203/I257-SpaA type |
| III     | 1                      | - - - | Koganei 65-0.15 |
| IVa     | 2                      | - - - | M203/I257-SpaA type |
| IVb-1   | 7                      | + - | M203/I257-SpaA type |
| IVb-2   | 14                     | + + + | M203/I257-SpaA type |

a) The lineages were determined based on genome-wide SNP analysis as previously described [7]; b) -, PCR negative; c) +, PCR positive.

### Table 2. Multiplex PCR results with various serovar *Erysipelothrix* strains from different host origins

| Strain | Serovar | Year of isolation | Origin | Multiplex PCR results with primers |
|--------|---------|-------------------|--------|------------------------------------|
| Kumamoto 13-1 | 1a | 2012 | Pig | + + + |
| Kagoshima 13-8 | 1a | 2012 | Pig | + + |
| Kagoshima 13-9 | 1a | 2012 | Pig | + + + |
| Kagoshima 13-10 | 1a | 2012 | Pig | + + |
| Kagoshima 13-11 | 1a | 2012 | Pig | + + |
| Kagoshima 13-13 | 1a | 2012 | Pig | + + |
| Kagoshima 13-17 | 1a | 2013 | Pig | + + |
| Hyogo 13-1 | 1a | 2013 | Pig | + + |
| Fukuoka 13-1 | 1a | 2013 | Pig | + + |
| Chiba 12-1 | 1a | 2012 | Pig | + + |
| Ibaraki 12-1 | 1a | 2012 | Pig | + + |
| Fuku 12-1 | 1a | 2012 | Pig | + + |
| Niigata 12-3 | 1a | 2012 | Pig | + + |
| Niigata 13-4 | 1a | 2013 | Pig | + + |
| Chiba 93-87 | 1b | 1993 | Pig | - - |
| Nagano 00-1 | 1b | 1999 | Pig | - - |
| Nagasaki 02-18 | 1b | 2002 | Pig | - - |
| Tokyo 03-2 | 1b | 2002 | Pig | - - |
| Oita 03-19 | 1b | 2002 | Pig | - - |
| Ishikawa 04-1 | 1b | 2003 | Pig | - - |
| Niigata 10-16 | 1b | 2010 | Pig | - - |
| Mie 11-5 | 1b | 2010 | Pig | - - |
| Kagoshima 13-13 | 1b | 2012 | Pig | - - |
| Niigata 13-5 | 1b | 2013 | Pig | - - |
| Niigata 94-2 | 2 | 1994 | Pig | - - |
| Iwate 95-4 | 2 | 1995 | Pig | - - |
| Mie 02-10 | 2 | 2000 | Pig | - - |
| Osaka 02-2 | 2 | 2002 | Pig | - - |
| Toyama 02-2 | 2 | 2002 | Pig | - - |
| Tochigi 02-1 | 2 | 2002 | Pig | - - |
| Yamanashi | 04-1 | 2 | 2004 | Pig | - - |
| Ishikawa 04-21 | 2 | 2004 | Pig | - - |
| Niigata 05-1 | 2 | 2003 | Pig | - - |
| Ishikawa 07-4 | 2 | 2004 | Pig | - - |
| Saitama 08-256 | 2 | 2008 | Pig | - - |
| Niigata 10-12 | 2 | 2008 | Pig | - - |
| Tottori 10-42 | 2 | 2010 | Pig | - - |
| Mie 11-1 | 2 | 2009 | Pig | - - |
| Nagano 11-2 | 2 | 2011 | Pig | - - |
| Niigata 12-8 | 2 | 2012 | Pig | - - |
| Kagoshima 13-1 | 2 | 2005 | Pig | - - |
| Kagoshima 13-18 | 2 | 2012 | Pig | - - |
| Kanagawa 13-A1 | 2 | 2009 | Pig | - - |

**E. rhusiopathiae** strains
Continuation of Table 2

| Straina) | Serovar | Year of isolation | Origin   | Multiplex PCR results with primers |
|----------|---------|-------------------|----------|-------------------------------------|
|          |         |                   |          | 0022F3 & 0022R3 | 0533F & 0533R | 0655F2 & 0655R1 |
| Hokkaido 13-B | 2 | 2013 | Pig | - | - | - |
| Hokkaido 14-B | 2 | 2012 | Pig | - | - | - |
| Ibaraki 09-4 | 5 | 2009 | Pig | - | - | - |
| Kanagawa 13-Y4 | 5 | 2013 | Pig | - | - | - |
| Kanagawa 13-Y2 | 6 | 2012 | Pig | - | - | - |
| Mie 13-14 | 11 | 2012 | Pig | - | - | - |
| Niigata 05-67 | 15 | 2004 | Pig | - | - | - |
| Aichi 12-1 | 19 | 2012 | Pig | - | - | - |
| Mie 02-47 | N | 1999 | Pig | - | - | - |
| Ishikawa 02-26 | N | 1999 | Pig | - | - | - |
| Ehime 02-38 | N | 2002 | Pig | - | - | - |
| Yamanashi 04-44 | N | 2003 | Pig | - | - | - |
| Mie 09-19 | Untypable | 2009 | Pig | - | - | - |
| Fujisawa | 1a | before 1972 | Pig | - | - | - |
| ME-7* | 1a | Unknown | Unknown | - | - | - |
| 422/1E* | 1b | 1958 | Pig | - | - | - |
| R32E11* | 2 | Unknown | Unknown | - | - | - |
| NF4E1* | 2 | 1958 | Pig | - | - | - |
| ATCC 19414T | 2 | Unknown | Pig | - | - | - |
| Doggerscharbe* | 4 | 1958 | Fish | - | - | - |
| Pécs 67* | 5 | 1970 | Pig | - | - | - |
| Tuzok* | 6 | 1970 | Bustard | - | - | - |
| Goda* | 8 | 1970 | Godwit | - | - | - |
| Kaparek* | 9 | 1962 | Fish | - | - | - |
| 14B | 9 | 1987 | Pen soil | - | - | - |
| IV.12/8* | 11 | 1964 | Pig | - | - | - |
| Pécs 9* | 12 | 1970 | Pig | - | - | - |
| Pécs 3597* | 15 | 1971 | Pig | - | - | - |
| Tanzania* | 16 | 1973 | Parrot | - | - | - |
| 545* | 17 | 1971 | Pig | - | - | - |
| 2017* | 19 | 1972 | Pig | - | - | - |
| Bâno 36* | 21 | Unknown | Sheep dip | - | - | - |
| CJPT-97* | 23 | Unknown | Pig | - | - | - |
| MEW22* | N | 1958 | Unknown | - | - | - |
| E. tonsillarum strains | | | | | | |
| Wittling* | 3 | Unknown | Fish | - | - | - |
| P-43* | 7 | 1960 | Fish | - | - | - |
| Lengyel-P* | 10 | Unknown | Squirrel | - | - | - |
| 2179 | 10 | 1977 | Pig slurry | - | - | - |
| Iszap-4* | 14 | 1970 | Mud of zoo pond | - | - | - |
| 2553* | 20 | 1975 | Pig | - | - | - |
| Bâno 107* | 22 | Unknown | Sheep dip | - | - | - |
| CJSF 14-2* | 24 | Unknown | Marine fish | - | - | - |
| KS20A* | 25 | Unknown | Pig slurry | - | - | - |
| L136* | 26 | Unknown | Pig slurry | - | - | - |
| Other sp.-1 | | | | - | - | - |
| Pécs 56* | 13 | 1970 | Pig | - | - | - |
| Other sp.-2 | | | | - | - | - |
| 715* | 18 | 1971 | Pig | - | - | - |

a) Serovar reference strains and the E. rhusiopathiae type strain are indicated with an asterisk and T, respectively; b) All serovar 1a strains, except for Fujisawa and ME-7, were determined by spaA genotyping as M203/I257-SpaA; c) +, PCR positive; d) -, PCR negative.

Two Lineage IVa strains isolated in 1994 and 2004 showed the same M203/I257-SpaA type as the Lineage IVb strains [7], thus indicating that, in addition to spaA genotyping, different genotyping methods are required to accurately identify recent strains belonging to the Lineage IVb strains for epidemiological studies of swine erysipelas in Japan.

Testing one hundred and twenty-seven strains of various serovars with different host origins, the SNP-based multiplex PCR assay...
could successfully detect and differentiate the *E. rhusiopathiae* strains belonging to either one of two clonal sublineages (Lineage IVb-1 and IVb-2), which are the predominant sublineages currently circulating in the pig population in Japan. Furthermore, the PCR assay accurately discriminated Lineage IVb strains from Lineage IVa strains with the same M203/I257-SpaA type. Lineage IVb-1 and IVb-2 independently evolved and spread separately on the Kyushu and Honshu islands, respectively[7]. Importantly, in the present study, two isolates from Hokkaido island were classified as Lineages IVb-1 and IVb-2 using multiplex PCR, and the classification results were indeed confirmed as correct by genome-wide SNP-based phylogenetic analysis (unpublished results). Additionally, a Honshu isolate (Hyogo 13-1) was identified as Lineage IVb-1 using multiplex PCR, and the result was confirmed by *spaA* genotyping. Thus, these results suggest that the Lineage IVb-1 and IVb-2 strains might be spreading across the country.

Finally, based on the genome-wide SNP data of the strains collected from various regions over two decades in Japan[7], we selected and utilized the SNP sites to design PCR primers. Interestingly, among the various serovar strains, the multiplex PCR detected only serovar 1a strains and successfully differentiated clonal sublineage strains currently circulating in the pig population in Japan. M203/I257-SpaA type strains are also emerging in China[7]. This multiplex PCR assay, which detects only recent M203/I257-SpaA type strains, may be useful for epidemiological studies of acute swine erysipelas in other eastern Asian countries.

**ACKNOWLEDGMENT.** This work was supported in part by NARO and the Ministry of Agriculture, Forestry and Fisheries of Japan.

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