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NOTE

Bacteriology

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.*

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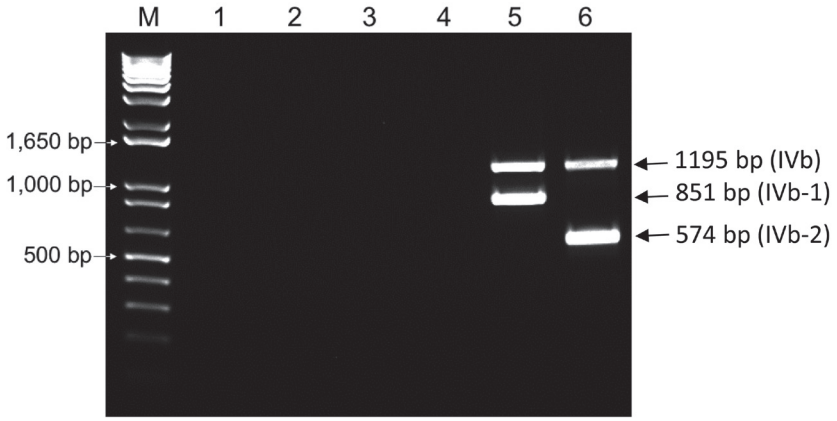

Fig. 1. Multiplex PCR for the simultaneous detection and differentiation of *E. rhusiopathiae* Lineage IVb-1 and IVb-2 strains. Lanes: 1, Lineage I strain (Nagano 98); 2, Lineage II strain (Akita 00); 3, Lineage III strain (Koganei 65-0.15); 4, Lineage IVa strain (Saitama 94); 5, Lineage IVb-1 strain (Miyazaki 11); 6, Lineage IVb-2 strain (Gifu 10). A molecular size marker (Invitrogen 1 Kb Plus DNA Ladder) is shown on the left (M).

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′-AATGACTATCGCAAAGAGCAAGGAAAACTG-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[a)]	No. of strains tested	Multiplex PCR results with primers			Remarks
		0022F3& 0022R3	0533F& 0533R	0655F2& 0655R1	
I	7	-[b)]	-	-	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	+[c)]	+	-	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[a)]	Serovar	Year of isolation	Origin	Multiplex PCR results with primers		
				0022F3& 0022R3	0533F& 0533R	0655F2& 0655R1
E. rhusiopathiae strains						
Kumamoto 13-1	1a[b)]	2012	Pig	+[c)]	+	-[d)]
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 13-15	1a	2011	Pig	+	+	-
Kagoshima 13-17	1a	2013	Pig	+	+	-
Kagoshima 13-20	1a	2011	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	+	-	+
Fukui 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 13-22	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	-	-	-

Continuation of Table 2

Strain[a)]	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 19414^T	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	-	-	-
Bãno 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	-	-	-
Bãno 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.

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