The Journal of Veterinary Medical Science

NOTE

Bacteriology

A PCR assay to specifically detect serovar 1a strains of *Erysipelothrix rhusiopathiae* and differentiate them from serovar 2 strains possessing an intact ERH_1440 gene

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J. Vet. Med. Sci.
84(1): 90–93, 2022
doi: 10.1292/jvms.21-0528

Received: 30 September 2021
Accepted: 6 November 2021
Advanced Epub:
18 November 2021

ABSTRACT. The *Erysipelothrix rhusiopathiae* ERH_1440 gene, which encodes CDP-glycerol:poly (glycerophosphate) glycerophosphotransferase, is conserved in serovar 1a strains. The gene is usually missing or truncated in other serovar strains and therefore has been used for PCR detection of serovar 1a strains. We have previously reported a rare case of an *E. rhusiopathiae* serovar 2 strain possessing an intact ERH_1440. In this study, we analyzed three additional serovar 2 strains with an intact ERH_1440 and developed a new PCR assay for the specific detection and differentiation of serovar 1a strains from these serovar 2 strains. PCR with primers designed based on serovar 1a-specific gene sequences upstream of ERH_1440 showed 100% specificity for four hundred thirty *Erysipelothrix* strains isolated from extensive origins.

KEY WORDS: ERH_1440, *Erysipelothrix rhusiopathiae*, PCR, serovar 1a, serovar 2

Erysipelothrix rhusiopathiae is a gram-positive bacterium and the major etiological agent of erysipelas in many species of mammals and birds, including swine, turkeys, and chickens [9]. Swine erysipelas, which may cause acute septicemia or chronic endocarditis and polyarthritis, is an important infectious disease resulting in enormous economic losses in the swine industry [9]. Among twenty-eight *Erysipelothrix* serovars, serovars 1 and 2 are most commonly associated with the disease in pigs, poultry, and humans [1–3, 10, 15]. In pigs, serovar 1a is usually isolated from acute septicemic cases, and serovar 2 is most common in subacute and chronic cases [9]; however, the molecular basis of these differences in virulence has not yet been fully clarified [6].

Recently, we reported a case of a pig that died from septicemia caused by infection of an *E. rhusiopathiae* serovar 2 strain, designated Ishikari, isolated in Hokkaido [14]. It was found that Ishikari is a very rare strain that was determined to be serovar 2 by a conventional gel double diffusion test but was PCR-positive for both serovars 1a and 2 (serovar 1a/2) by a serotyping PCR assay [13]. Sequence analysis revealed that the Ishikari strain possesses an intact ERH_1440 gene, which is conserved in serovar 1a strains and usually missing or truncated in other serovar strains. In this study, to develop a new PCR system to detect only *E. rhusiopathiae* serovar 1a strains, we analyzed three additional swine isolates that were determined to be serovar 1a/2 by serotyping PCR assay [13]. The field isolates were independently isolated from the kidney of a miscarried fetus, an arthritic lesion of a growing-finishing pig, and the heart from a pig that died from septicemia collected from different farms in Hokkaido. Serotyping of the isolates was performed by a double agar-gel precipitation test with heat-stable peptidoglycan antigens and type-specific rabbit antisera, and three isolates were determined to belong to serovar 2. We sequenced the ERH_1440 gene of the serovar 2 strains by the Sanger method. Briefly, a DNA fragment containing the ERH_1440 gene was amplified and sequenced using the primers 1438F (5′- CAAAGATTGTAAGTCCACGC-3′) and 1441R2 (5′-GGGCTGCTTACTACCCATAC-3′), which correspond to nucleotide positions 318 to 337 of ERH_1438 and 1022 to 1041 of ERH_1441, respectively. The unsequenced internal region was further sequenced using the amplified DNA fragment as the template with primers 1440R (5′-TGATCCTTTGCCACTAATGC-3′) and 1440R2 (5′-GCGATAGGGTATTCACGCAC-3′), which correspond to nucleotide positions 984 to 1003 and 367 to 386 of the ERH_1440 gene, respectively.

Sequence analysis revealed that ERH_1440 genes of these serovar 2 strains have 99–100% nucleotide sequence identity with those of the Fujisawa strain (serovar 1a) (accession no. AP012027) [5, 6] and Ishikari strain (serovar 2) (accession no. LC556333) [14]. The hypervariable region sequence of *spaA* (*s*urface *p*rotective *a*ntigen) has been used to examine the genetic diversity of

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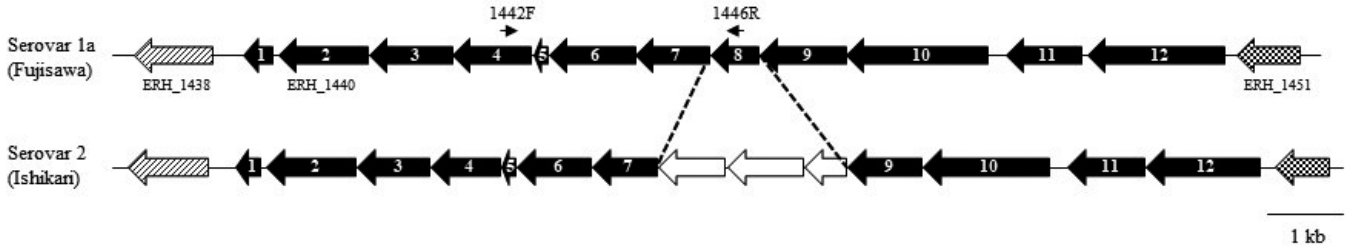

Fig. 1. Schematic representation of the chromosomal region defining the antigenicity of the *Erysipelothrix rhusiopathiae* strains Fujisawa (serovar 1a) and Ishikari (serovar 2). Identical genes are indicated by the same numbers. Small arrows indicate the locations of the primer pairs, 1442F and 1446R, used in this study.

E. rhusiopathiae field isolates [4, 16]. It has also been reported that *spaA* genotyping of *E. rhusiopathiae* strains is a practical alternative to whole-genome sequencing analysis of the *E. rhusiopathiae* isolates from eastern Asian countries [7]. To examine whether these serovar 2 strains are clonal, we sequenced the *spaA* gene as previously described [11]. Sequence analysis of the hypervariable region at positions 502 to 933 nucleotides (432 bp) of *spaA* from these strains showed 100% nucleotide sequence identity with Ishikari and single nucleotide polymorphisms (SNPs) were observed at various different positions in the repeat sequences encoding the choline-binding domain in the C-terminus (data not shown). Thus, these isolates showed the same *spaA* type, suggesting that they may be clonal strains and currently circulating in Hokkaido. However, to clarify the hypothesis, whole-genome sequencing analysis will be necessary.

To specifically detect and differentiate serovar 1a and these serovar 2 strains, primers were designed based on serovar-specific gene sequences of the reference *E. rhusiopathiae* Fujisawa genome (accession no. AP012027) [5, 6]. The primer sequences, 1442F (5′-CGTGTATTTTCAATCGTGGACACCA-3′) and 1446R (5′-TAAAGCCTGGATTGACTGGG-3′), which correspond to nucleotide positions 326 to 350 of ERH_1442 and 419 to 438 of ERH_1446, respectively (Fig. 1), were designed to amplify a 2,868-bp fragment using the *in silico* Molecular Cloning Genomics Edition (IMCGE) software [8].

The specificity of the primers was assessed empirically with other serovar strains and then validated by testing the serovar 2 strains possessing an ERH_1440. PCR was performed using a BIO-RAD T-100 thermal cycler (BIO-RAD, Hercules, CA, USA) with the following conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, 58°C for 30 sec, and 68°C for 3 min. The reaction mixtures (25 µl) contained 50 ng of template DNA, 0.2 µM of each primer, and 12.5 µl of 2 × Quick Taq HS DyeMix (TOYOBO, Osaka, Japan).

The specificity of 1442F and 1446R primers was eventually tested with four hundred thirty *Erysipelothrix* species strains isolated from different host origins. The results showed 100% specificity for all strains tested (Table 1), showing high specificity of the assay. Figure 2 shows an example of the PCR results with serovar 1a, 1b, 2, and 5 strains, the last of which is often isolated from wild animals.

In this study, we developed a PCR assay that can differentiate *E. rhusiopathiae* serovar 1a strains from serovar 2 strains possessing an intact ERH_1440 gene. The ERH_1440 gene encoding CDP-glycerol:poly (glycerophosphate) glycerophosphotransferase is a paralog of ERH_0432, which is an important gene involved in capsule expression and virulence of the organism [12]. Among the serovar 2 strains tested in this study, two strains were isolated from a pig that died from septicemia and a miscarried fetus, suggesting that these strains are as highly virulent to pigs as serovar 1a strains. The Ishikari strain reported in our previous study was isolated from an acute case [14]. Taken together, the findings in this study support the hypothesis that serovar 2 strains possessing an intact ERH_1440 gene may be more virulent than serovar 2 strains without the gene. In this regard, a serotyping PCR assay [13], which can differentiate serovar 1a, 1b, 2, and 5 strains of *E. rhusiopathiae*, and the PCR assay developed in this study will be useful for epidemiological studies of the infection.

CONFLICT OF INTEREST. The authors declare no potential conflicts of interest.

ACKNOWLEDGMENT. This work was supported by a grant from the National Agriculture and Food Research Organization.

Table 1. Validation of PCR with various serovar *Erysipelothrix* strains from different host origins

Serovar	Origin	No. of PCR positive strains/No. of strains tested
E. rhusiopathiae		
1a	Pig	155/155
	Chicken	1/1
	Unknown	1/1
1b	Pig	0/38
	Human	0/1
	Chicken	0/1
	Wild boar	0/4
2	Pig	0/123
	Human	0/1
	Dolphin	0/1
	Wild boar	0/7
	Raccoon	0/1
	Unknown	0/1
2/15 [a]	Pig	0/1
	Wild boar	0/2
2/21 [b]	Pig	0/3
	Crow	0/4
	Human	0/1
4	Fish	0/1
	Pig	0/5
5	Pig	0/6
	Mud of pig farm	0/1
	Wild boar	0/2
	Chicken	0/1
	Crow	0/1
	Raccoon	0/1
	Raccoon dog	0/1
6	Pig	0/4
	Dolphin	0/2
	Crow	0/1
	Bustard	0/1
8	Wild boar	0/2
	Godwit	0/1
	Pig	0/2
9	Fish	0/1
	Pen soil	0/1
	Pig	0/1
11	Pig	0/12
	Wild boar	0/1
12	Pig	0/2
15	Pig	0/1
16	Parrot	0/1
17	Pig	0/2
19	Pig	0/2
21	Sheep dip	0/1
	Pig	0/3
	Wild boar	0/1
23	Pig	0/1
N	Pig	0/2
Untypable	Pig	0/3
	Penguin	0/1
	Raccoon dog	0/1
	Human	0/1
E. tonsillarum		
3	Fish	0/1
7	Fish	0/1
	Pig	0/1
10	Squirrel	0/1
14	Mud of zoo pond	0/1
20	Pig	0/1
22	Sheep dip	0/1
24	Marine fish	0/1
25	Pig slurry	0/1
26	Pig slurry	0/1
Erysipelothrix sp. strain 2		
18	Pig	0/1
10	Pig slurry	0/1
Erysipelothrix sp. strain 1		
13	Pig	0/1
E. inopinata		
NT [c]	Vegetable broth	0/1

[a] Reactive with both serovar 2 and 15 antisera. [b] Reactive with both serovar 2 and 21 antisera. [c] Not tested.

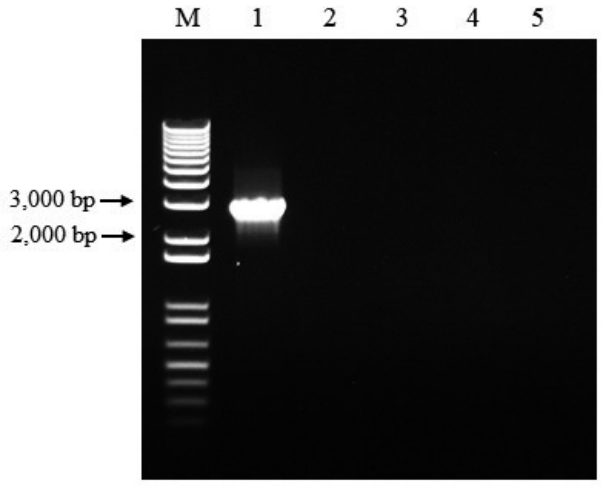

Fig. 2. PCR assay to differentiate *Erysipelothrix rhusiopathiae* serovar 1a strains and serovar 2 strains possessing an intact ERH_1440 gene. Lanes: 1, Fujisawa strain (serovar 1a); 2, 422/1E strain (serovar 1b); 3, R32E11 strain (serovar 2); 4, Pécs 67 strain (serovar 5), and 5, Ishikari strain (serovar 2). A molecular size marker (1-kb Plus DNA ladder; Invitrogen) is shown to the left (M).

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