# Epidemiological studies on pathogenic *Yersinia* in wild rodents and development of rapid detection methods for those pathogens

# ノネズミにおける病原性エルシニアに関する疫学的研究ならびにこれら病原体の迅速検出法の開発

2022

Cooperative Division of Veterinary Sciences,

Graduate School of Agriculture,

Tokyo University of Agriculture and Technology

BUI Thi Hien

**CONTENTS**

# PREFACE

*Yersinia* is gram-negative, facultative anaerobic bacteria, belongs to the family *Yersiniaceae* [Carniel et al., 2006, Adeolu et al., 2016]. The genus *Yersinia* consists of 28 species [Murros-Kontiainen et al., 2011, Nguyen et al., 2019, 2020, 2021, Le Guern et al., 2020]. Among them, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis,* are known to cause yersiniosis [Carniel et al., 2006, Galindo et al., 2011]. Yersiniosis is human foodborne and zoonotic disease [Bari et al., 2011, Galindo et al., 2011, Fredriksson-Ahomaa, 2017]. It was the third most frequently reported foodborne zoonotic disease in the European countries in 2017 [EFSA and ECDC, 2018]. Centers for Diseases Control and Prevention (CDC) estimates that *Yersinia* causes almost 117,000 illnesses, 640 hospitalizations, and 35 deaths in USA every year [CDC, 2018]. Generally, pathogenic *Yersinia* causes gastroenteritis with clinical symptoms such as abdominal pain, diarrhea, and fever; however, highly pathogenic *Y. enterocolitica* serotypes and *Y. pseudotuberculosis* sometimes cause septicemia [Bottone, 1997, Hosaka et al., 1997, Fredriksson-Ahomaa, 2017, Hashimoto et al., 2021]. From over 60 *Y. enterocolitica* serotypes, only 9 serotypes (O3, O4,32, O5,27, O8, O9, O13, O18, O20, and O21) are pathogenic to humans [Bottone, 1997, 1999, Fredriksson-Ahomaa, 2007]. Among them, serotypes O3, O5,27, and O9 show low pathogenicity to humans. In contrast, the remaining 6 serotypes, O4,32, O8, O13, O18, O20, and O21, which are called “American strains”, show high pathogenicity to humans [Bottone, 1997, 2015, Carniel et al., 2006]. On the other hand, *Y. pseudotuberculosis* is classified into 21 serotypes, and various serotypes (1b, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 10, and 15) are isolated from human patients [Fukushima et al., 2001b, Amphlett, 2015, Hashimoto et al., 2021].

*Y. enterocolitica* and *Y. pseudotuberculosis* are widely distributed in mammals, birds, and the environment [Carniel et al., 2006, Fredriksson-Ahomaa, 2007]. Many human yersiniosis cases can be transmitted from animal reservoirs (farm animal, wild animal, etc.) directly via feces or indirectly through food and the environment [Fredriksson-Ahomaa, 2007, 2017, Galindo et al., 2011]. Some researchers suggested that wild rodents are the natural reservoirs for pathogenic *Yersinia* and the source of human yersiniosis [Fukushima et al., 1990, Hayashidani et al., 1995, Oda et al., 2015, Joutsen et al., 2017, Platt-Samoraj et al., 2020]. Fukushima [1991] challenged *Y. pseudotuberculosis* 1b and 4b intragastrically to large Japanese field mice (*Apodemus speciosus*) and reported that this wild rodent species showed susceptible to pathogenic *Y. pseudotuberculosis* 1b and 4b, and shed these pathogens through their feces for 1-2 weeks after challenging. Hayashidani et al. [1994] also challenged pathogenic *Y. enterocolitica* O8 intragastrically to 3 species of wild rodents, including *A. speciosus*, small Japanese field mice (*A. argenteus*) and Anderson's red-backed voles (*Eothenomys andersoni*), and found that these 3 rodent species infected with *Y. enterocolitica* O8 and shed this pathogen in their feces for more than 2 weeks. The wild rodent species such as *A. speciosus* and *A. argenteus,* are commonly distributed in mountainous areas in Japan [Nakata et al., 2009]. Pathogenic *Y. pseudotuberculosis* was isolated from wild rodents in Shimane Prefecture in the western part of Honshu Island [Fukushima et al., 1990], and Hokkaido Island in northern part of Japan [Kaneko and Hashimoto, 1981]. On the other hand, pathogenic *Y. enterocolitica* O8 was isolated from wild rodents in Niigata Prefecture and Aomori Prefecture, located in the eastern part of Honshu Island, Japan [Iinuma et al., 1992, Hayashidani et al., 1995, Oda et al., 2015]. Fukushima Prefecture is located in the eastern part of Honshu Island, and is next to Niigata Prefecture. However, prevalence of pathogenic *Yersinia* in wild rodents living in Fukushima Prefecture has not been reported. Moreover, the ecology and epidemiology of pathogenic *Yersinia* in wild rodents is still unclear.

*Y. enterocolitica* and *Y. pseudotuberculosis* are known to be distributed worldwide [Carniel et al. 2006, Galindo et al., 2011]. In Japan, human *Y. pseudotuberculosis* and *Y. enterocolitica* infections have been reported [Hayashidani, 2016, Minami et al., 2017, Hashimoto et al., 2021]. Among pathogenic *Y. enterocolitica*, serovar O8, belonging to "American strains", shows highly pathogenic to humans [Bottone, 1997, 2015, Carniel et al., 2006]. The geographical distribution of this serovar was very limited. It had been isolated from humans and animals only in North America until 1989 [Iinuma et al., 1992, Botton, 1999, 2015]. Iinuma et al. [1992] isolated this serotype from wild rodents living in Niigata Prefecture. This was the first isolation of this serovar in Japan. After that, this serovar has been isolated from humans and wild rodents in Japan [Ichinohe et al., 1991, Saitoh et al., 1994, Hayashidani et al., 1995, Oda et al., 2015, Konishi et al., 2016, Minami et al., 2017]. Recently, this serotype has been isolated from human patients in European countries such as Germany, Poland, and France [Rastawicki et al., 2009, Rosner et al., 2010, Savin et al., 2018; Wallet et al., 2020], although natural reservoir of this serotype has not been identified in this region [Fredriksson-Ahomaa, 2017]. Therefore, the medical doctor is required to know which type of pathogenic *Yersinia* infects human yersiniosis patients immediately. The diagnostic methods for pathogenic *Yersinia* are mainly based on the culture method. It takes about 3 weeks when cold enrichment culture method is used. However, it is time-consuming and laborious [Fukushima et al., 2011, Konishi et al., 2016, Fredriksson-Ahomaa, 2017]. Polymerases chain reaction (PCR) methods are considered more rapid and sensitive detection method for pathogenic *Yersinia* rather than culture method [Boyapalle et al., 2001, Fredriksson-Ahomaa and Korkeala, 2003, Fredriksson-Ahomaa et al., 2006]. Some PCR methods have been developed for the detection of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* together [Fredriksson-Ahomaa and Korkeala, 2003, Foley et al., 2019]. However, no PCR methods have been developed for simultaneous detection and

differentiation of the highly pathogenic *Y. enterocolitica,* low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis*.

The main objectives of this present study were to clarify the ecology and epidemiology of pathogenic *Yersini*a, especially *Y. enterocolitica* O8 in wild rodents in Japan and to develop rapid, sensitive, and specific PCR methods to detect and distinguish 3 pathogenic *Yersinia* groups such as highly pathogenic *Y. enterocolitica,* including serotype O8, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis.*

In Chapter 1, the prevalence of pathogenic *Yersinia* in wild rodents in Fukushima Prefecture was studied to know the ecology and epidemiology of pathogenic *Yersinia* in wild rodent populations.

In Chapter 2, a rapid and specific multiplex PCR method for detecting and differentiating 3 pathogenic *Yersinia* groups such as highly pathogenic *Y. enterocolitica,* including serotype O8, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis* was developed.

In Chapter 3, SYBR Green and TaqMan multiplex real-time PCR methods for more rapid and sensitive diagnosis, differentiating 3 pathogenic *Yersinia* groups were developed. The ability and usefulness of the developed SYBR Green and TaqMan multiplex real-time PCR methods were compared with the multiplex PCR method developed in Chapter 2.

# CHAPTER 1

## Prevalence of pathogenic *Yersinia* in wild rodents in Fukushima Prefecture, Japan

## 1.1. INTRODUCTION

In 1991, the first human *Y. enterocolitica* O8 infection was reported in Aomori Prefecture, Japan [Ichinohe et al., 1991]. After that, human *Y. enterocolitica* O8 infection cases have been reported. Moreover, outbreaks of human *Y. enterocolitica* O8 infection were recorded in some prefectures such as Nara Prefecture [Sakai et al., 2005], Toyama Prefecture [Isobe et al., 2014], Tokyo Metropolitan [Konishi et al., 2016], and Nagano Prefecture [Yoshida et al., 2017]. However, the sources of *Y. enterocolitica* O8 infections were not identified in those outbreaks [Konishi et al., 2016, Yoshida et al., 2017]. On the other hand, some researchers isolated *Y. enterocolitica* O8 from wild rodents, and indicated that wild rodents are natural reservoir of this pathogen in Japan [Iinuma et al., 1992, Hayashidani et al., 1995, Oda et al., 2015]; however, the role of wild rodents in the ecology and epidemiology of *Y. enterocolitica* O8 is still unknown.

In Chapter 1, we examined for the prevalence of pathogenic *Yersinia* in wild rodents living in Fukushima Prefecture at the same location for a long term to clarify the ecology and epidemiology of this pathogen in nature.

## 1.2. MATERIALS AND METHODS

### 1.2.1. Sample collection

From July 2012 to April 2021 (total 19 times), a total of 755 wild rodents, 464 large Japanese field mice (*Apodemus speciosus*), 232 small Japanese field mice (*Apodemus*

*argenteus*), 37 Japanese grass voles (*Microtus montebelli*), and 22 Japanese shrew moles (*Urotrichus talpoides*) were captured in the mountainous areas of Nihonmatsu city in Fukushima Prefecture located in the eastern part of Honshu Island, Japan. There are 2 mountains in this area, Mt. Kuchibuto (37°36'N, 140°34'E) and Mt. Hayama (37°33'N, 140°37'E). These mountains are 3 km apart. Wild rodents were captured twice a year, that were mainly in spring (April) and autumn (November) except in 2012 (July) and 2020 (June). Wild rodents were captured at the same points during the survey period (Figure 1.1). Wild rodents were captured by live traps. Traps were set in the afternoon before sunset and checked early the following morning. After euthanasia by cervical dislocation method [Science Council of Japan, 2006, Gannon and Sikes, 2007], the rodents were preserved separately in sterile plastic bags and immediately transported to the Laboratory of Animal Health, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu city, Tokyo, Japan, under refrigeration condition (2–7$^{o}$C) in the cold containers. Each wild rodent was measured, classified by species, sex, and age, and then dissected to collect rectal content samples. The age of *A. speciosus* was determined based on the body weight by the method of Fukushima et al. [1990]. Briefly, the rodents that weighed under 30 g or over 30 g were divided into juveniles or adults, respectively. Almost all other species of wild rodents captured were adults. Sampling procedures followed the Regulations for Animal Experiments at Tokyo University of Agriculture and Technology and were approved by the institution's Committee for Animal Research and Welfare.

### 1.2.2. *Yersinia* isolation and identification

The cold enrichment culture method was applied to isolate pathogenic *Yersinia* from wild rodent fecal samples. The rectal contents (ca 0.5 g) of animals were homogenized in 9

times amount of phosphate-buffered saline (PBS; pH 7.2) and incubated at 4ºC for 3–4 weeks [Iwata et al., 2005]. After alkali (KOH) treatment [Aulisio et al., 1980], the sample suspension was then cultured on irgasan-novobiocin (IN) [Fukushima et al., 1990] and CHROMagar Yersinia (Currently CHROMagar Y.enterocolitica) (CHROMagar Microbiology, Paris, France) plates and incubated at 25ºC for 48 h. After that, 4 morphologically suspicious colonies from each selective agar were picked up and subcultured onto trypticase soy agar (TSA, Becton, Dickinson and Co., Franklin Lakes, NJ, USA). *Yersinia* identification was accomplished by the method described previously [Iwata et al., 2005]. Serotyping of *Yersinia* strains isolated from wild rodents was performed by slide agglutination with commercial rabbit anti-*Y. enterocolitica* sera and rabbit anti-*Y. pseudotuberculosis* sera (Denka-Seiken, Tokyo, Japan). All *Yersinia* isolates were examined for their temperature-dependent autoagglutination by the method of Laird and Cavanaugh [1980] and the presence of the virulence plasmid by the modified method of Kado and Liu [1981] to evaluate their potential pathogenicity. The pathogenic *Yersinia* virulence genes, including *fyu*A, *ail, inv,* and *virF* of *Yersinia* isolates were detected using multiplex PCR method developed in Chapter 2.

### 1.2.3. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed to compare the genetic characteristics of *Y. enterocolitica* O8 isolates. The PFGE was carried out as described by Iwata et al. [2005]. Briefly, chromosomal DNAs were digested by the restriction enzyme *Not*I (Takara Bio Inc., Kusatsu, Japan) for 3 h at 37ºC. The DNA fragments were separated in 1.2% agarose NA (GE healthcare, Bioscience AB, Uppsala, Sweden) on a CHEF-DRII Pulsed Field Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Electrophoresis was carried out for 24 h at 14ºC and 200 V with pulse times of 2 to 25 sec. A CHEF DNA Size Standard Lambda Ladder (Bio-

Rad) was used as molecular size marker. The gels were stained with AtlasSight DNA Stain (Bioatlas, Tartu, Estonia) and photographed using a Gel Doc camera system (Bio-Rad).

### 1.2.4. Statistical analysis

Differences in the prevalence were analyzed by Fisher's exact test using R statistical software, R version 4.0.5, 2021 [R Core Team, 2021].

## 1.3. RESULTS

### 1.3.1. Prevalence of pathogenic *Yersinia* in wild rodents in Fukushima Prefecture

Thirteen *Y. enterocolitica* O8 and 1 *Y. pseudotuberculosis* 5b strains were isolated from 14 (1.9%) of 755 wild rodents captured in Fukushima Prefecture from 2012 – 2021 (Table 1.1). All *Y. enterocolitica* O8 isolates harbored virulence genes such as *fyuA, ail,* and *virF*. However, the *Y. pseudotuberculosis* 5b isolate did not harbor *virF* gene. Therefore, this isolate was identified as non-pathogenic *Y. pseudotuberculosis*. For 10-year observations, the *Y. enterocolitica* O8 was isolated from *A. speciosus* and *A. argenteus* in 6 of 19 times surveys. This pathogen was isolated from both *A. speciosus* and *A. argenteus* at the same opportunity of capture in April 2016 and June 2020. No *Yersinia* isolate was recovered from *M. montebelli* and *U. talpoides*. No significant difference in the prevalence of *Y. enterocolitica* O8 among seasons was observed.

Of 13 pathogenic *Y. enterocolitica* O8 positive rodents, 6 (1.2%) were from 464 *A. speciosus*, and 7 (3.0%) were from 232 *A. argenteus* (Table 1.2). No significant difference in the isolation rate of *Y. enterocolitica* O8 between *A. speciosus* and *A. argenteus* was observed. Of 6 *Y. enterocolitica* O8 positive *A. speciosus*, 3 were females and 3 were males. Moreover, of 6 those *A. speciosus*, 1 female and 2 males were juveniles. Of 3 *Y. enterocolitica* O8 positive juvenile, 1 was captured in Mt. Hayama and 2 were captured in Mt. Kuchibuto. No significant differences in *Y. enterocolitica* O8 isolation rate were observed among sex in both rodent species.

### 1.3.2. The genetic characteristics of *Y. enterocolitica* O8 isolates

Digestion of genomic DNA from 13 *Y. enterocolitica* O8 isolates with *Not*I gave 21 to 22 fragments (Figure 1.2). Of 13 *Y. enterocolitica* O8 isolates, strain YE15-29 isolated in 2015 showed the PFGE pattern P1, and the other 12 *Y. enterocolitica* O8 isolates showed the same PFGE pattern P2. However, patterns P1 and P2 differ in only 1 band. P1 and P2 were identified as the same clone, following the guideline of Tenover et al. [1995].

## 1.4. DISCUSSION

In the present study, *Y. enterocolitica* O8 had been frequently isolated from wild rodents living in Fukushima Prefecture although non-pathogenic *Y. pseudotuberculosis* 5b was isolated only 1 time. The pathogenic *Y. enterocolitica* O8 was isolated from wild rodents living in Niigata and Aomori Prefecture, located eastern part of Honshu Island of Japan

[Iinuma et al., 1992, Hayashidani et al., 1995, Oda et al., 2015]. On the other hand, Fukushima et al. [1990] reported that pathogenic *Y. pseudotuberculosis* 1b and 4b were isolated from wild rodents in Shimane Prefecture, located western part of Honshu Island of Japan. Moreover, pathogenic *Y. pseudotuberculosis* was detected from a wild rodent in Hokkaido Island of Japan [Kaneko and Hashimoto, 1981]. Fukushima et al. [2001a] also reported that the wild rodents which harbored *Y. enterocolitica* O9 in China are protected against *Y. pestis* infection. Until now, no report on the prevalence of pathogenic *Yersinia* in wild rodents has been reported in Fukushima Prefecture although pathogenic *Y. enterocolitica* O8 is known to be distributed in eastern part of Honshu Island of Japan. This is the first report of the isolation of pathogenic *Y. enterocolitica* O8 in wild rodents living in this Prefecture.

Almost all researchers have tried to isolate pathogenic *Yersinia* in wild rodents to know the prevalence of this pathogen 1- or 2-times surveys [Iinuma et al., 1992, Hayashidani et al., 1995, Oda et al., 2015]. We observed the prevalence of pathogenic *Yersinia* in wild rodent populations in Fukushima Prefecture for 10 years. For 10-year observations, *Y. enterocolitica* O8 was isolated from wild rodents in 6 of 19 times surveys. A total of 13 pathogenic *Y. enterocolitica* O8 isolates were detected from wild rodents during those periods. All 13 *Y. enterocolitica* isolates showed the same PFGE patterns. Those results indicated that the same clone of *Y. enterocolitica* O8 has been maintained in wild rodent populations in this area for at least 6 years. Generally, pathogenic *Yersinia* seems to show “habitat isolation” in wild rodents in the world [Mollaret, 1995]. This phenomenon, “habitat isolation”, means that 1 wild rodent population usually maintain 1 pathogenic *Yersinia* species although the mechanism of this phenomenon is still unknown. Therefore, the wild rodent populations in this survey area of Fukushima Prefecture seem to maintain only *Y. enterocolitica* O8. Moreover, the *Y. enterocolitica* O8 was isolated from wild rodents captured in Mt. Hayama and Mt. Kuchibuto at the same time in 2016, 2020 and 2021, and showed the same genetic

type. Two mountains, Mt. Hayama and Mt. Kuchibuto, are 3 km apart from each other, and wild rodents may migrate among both mountains although wild rodents' sphere of activity is not usually wide except breeding season [Kuroda and Katsuno, 2007]. Furthermore, Hayashidani et al. [1994] challenged pathogenic *Y. enterocolitica* O8 to *A. speciosus* intragastrically, and clarified that *A. speciosus* infected with this pathogen and shed this pathogen throught there feces for more than 2 weeks after challenging. The wild rodent's feces including pathogenic *Yersinia* might contaminate the environment such as feeds, soil, and water. Those results indicated that horizontal and vertical transmission of pathogenic *Y. enterocolitica* O8 seem to occur among wild rodent populations. Further studies are needed to clarify clearly the ecology of pathogenic *Y. enterocolitica* O8 in wild rodent populations.

In this study, the pathogenic *Y. enterocolitica* O8 strains were isolated from *Apodemus* species but not from *M. montebelli* and *U. talpoides,* although they were captured in the same areas. Some researchers reported that pathogenic *Yersinia* such as *Y. enterocolitica* and *Y. pseudotuberculosis* were isolated from *Apodemus* species but not from *M. montebelli* and *U. talpoides* [Fukushima et al., 1990, Hayashidani et al., 1995, Oda et al., 2015]. Fukushima [1991] and Hayashidani et al. [1994] found that *A. speciosus* showed susceptibility to pathogenic *Y. pseudotuberculosis* or *Y. enterocolitica* O8 respectively. However, there is no data related to the susceptibility of *M. montebelli* and *U. talpoides* to pathogenic *Yersinia*. Further research should be done to clarify the susceptibility of *M. montebelli* and *U. talpoides* to pathogenic *Yersinia*.

## 1.5. SUMMARY

From 2012 to 2021, prevalence of pathogenic *Yersinia* in wild rodents captured in Fukushima Prefecture, Japan was investigated twice a year to clarify the ecology of this pathogen in wild rodent populations. A total of 755 wild rodents, including 464 large Japanese field mice (*Apodemus speciosus*), 232 small Japanese field mice (*Apodemus argenteus*), 37 Japanese grass voles (*Microtus montebelli*) and 22 Japanese shrew moles (*Urotrichus talpoides*), were captured. Pathogenic *Yersinia enterocolitica* O8 was isolated from 13 (1.7%) of 755 wild rodents. All *Y. enterocolitica* O8 isolates were pathogenic strains, harboring 3 virulent genes, including *ail, fyuA*, and *virF*. Of 13 pathogenic *Y. enterocolitica* O8 positive rodents, 6 (1.2%) were from 464 *A. speciosus*, and 7 (3.0%) were from 232 *A. argenteus*. No *Y. enterocolitica* O8 isolate was recovered from *M. montebelli* and *U. talpoides*. This pathogen was isolated repeatedly from wild rodents in April 2015, 2016, and 2017, in June and November 2020, and in April 2021, which was 6 of 19 times of observations. All *Y. enterocolitica* O8 isolates showed the same PFGE patterns. These results indicated that the same clone of pathogenic *Y. enterocolitica* O8 has been maintained in wild rodent populations in Fukushima Prefecture. Therefore, wild rodent populations contribute substantially to the continuous transmission of *Y. enterocolitica* O8 and its persistence in the ecosystem. This is the first report on the isolation of pathogenic *Y. enterocolitica* O8 in wild rodents in Fukushima Prefecture, Japan.

# CHAPTER 2

## Development of multiplex PCR method for differentiating highly pathogenic *Yersinia enterocolitica*, low pathogenic *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*

## 2.1. INTRODUCTION

A few multiplex PCR methods have been developed to detect both pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* [Nakajima et al., 1992, Weynants et al., 1996, Kot et al., 2007, Stenkova et al., 2008]. Three groups of pathogenic *Yersinia,* including highly pathogenic *Y. enterocolitica,* low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis*, seem to cause yersiniosis in Japan [Hayashidani, 2016, Minami et al., 2017, Hashimoto et al., 2021]. However, the multiplex PCR method for rapid detection and differentiation of 3 groups of these pathogen has not been developed.

In this Chapter, we developed multiplex PCR method for diagnosis and differentiation of highly pathogenic *Y. enterocolitica,* low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis,* and evaluated the performance of the method in the detection of pathogenic *Y. enterocolitica* O8 from clinical samples.

## 2.2. MATERIALS AND METHODS

### 2.2.1. Bacteria strains

A total of 25 strains of pathogenic *Yersinia,* including 6 strains of low pathogenic *Y. enterocolitica* serotypes, 9 strains of highly pathogenic serotypes, and 10 strains of pathogenic *Y. pseudotuberculosis* were used in this study. Moreover, non-pathogenic *Y. enterocolitica* serotype O8,19*, Y. aldovae, Y. intermedia, Y. kristensenii, Y. rohdei*, *Escherichia coli*, and *Salmonella enterica* subsp. *enterica* serovar Enteritidis were used to verify the specificity of

the multiplex PCR method (Table 2.1). Almost all reference strains except 1 highly pathogenic *Y. enterocolitica,* serotype O8, were described in previous study [Horisaka et al., 2004]. These strains were stored in skim milk at -80ºC until analysis.

### 2.2.2. DNA extraction from bacteria

All bacterial strains were plated on TSA and incubated for 24 h at 25ºC. After suspending the bacterial cells of each strain in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), genomic DNA was extracted by using the alkali-heat DNA extraction method described previously [Hara-Kudo et al., 2016]. Briefly, 200 µl of the bacterial suspension was centrifuged at 10,000 ×*g* for 10 min. The collected pellet was resuspended in 85 µl of sterilized 50 mM NaOH, followed by heating at 100°C for 10 min. After cooling on ice, the suspension was neutralized with 15 µl of sterilized 1 M Tris-HCl (pH 7.0) and then was centrifuged at 10,000 ×*g* for 10 min. The supernatant containing the DNA template was collected and used for the multiplex PCR.

### 2.2.3. Primer selection

The target genes were selected based on their ability to identify all pathogenic *Yersinia*, including highly pathogenic *Y. enterocolitica*, low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis*. These genes included *fyuA* (ferric yersiniabactin uptake receptor A), present on chromosomal DNA of highly pathogenic *Y. enterocolitica* [Rakin et al., 1995, Savin et al., 2018]; *ail* (attachment invasion locus), found uniquely on the chromosome of pathogenic *Y. enterocolitica* strains [Miller et al., 1989, Thoerner et al., 2003]; *inv* (invasion),

present on the chromosome of pathogenic *Y. pseudotuberculosis* [Isberg et al., 1987, Thoerner et al., 2003]; and *virF* (virulence regulon transcriptional activator of the *Yersinia*), encoded on a 70 kilobase plasmid (pYV) of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* [Carniel et al., 2006, Bancerz-Kisiel et al., 2018]. PCR primers targeting the *fyuA* gene were designed for this study. A region of the *fyuA* gene sequence of *Y. enterocolitica* serotype O8 (GenBank accession no. Z35486.1) which lacks homology with the *fyuA* gene sequence of *Y. pseudotuberculosis* was chosen to design *fyuA* gene-specific primers using the Primer-BLAST software [Ye et al., 2012]. The primer pairs for *inv, ail,* and *virF* were designed by Thoerner et al. [2003]. The details of primers specific to each target genes are shown in Table 2.2.

### 2.2.4. Multiplex PCR method

Initially, monoplex PCR using each primer pair was performed to observe the distribution of target genes among pathogenic *Yersinia* species. After validation of each pair, these 4 primer pairs were combined to confirm that each PCR product was the correct size. Subsequently, the multiplex PCR conditions were optimized. Each reaction mixture (12 µl) contained 0.1 µM of each primer, 1X Green buffer of Gotaq Flexi DNA polymerase kit (Promega Corp., Madison, WI, USA), 2.5 mM $MgCl_2$, 200 µM dNTP, 0.05 U Gotaq DNA polymerase (Promega), and 2 µl of template DNA. The reaction was performed in a T100 Thermal Cycler (Bio-Rad) under the following conditions: initial denaturation step for 2 min at 95°C, followed by 30 cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 5 min. The PCR products were then subjected to electrophoresis on 1.5 % agarose ME (Fujifilm Wako Pure Chemical Corp., Osaka, Japan) gel in 1X Tris-acetic acid EDTA buffer (Fujifilm Wako Pure Chemical Corp.) at 100 V for 30 min and stained with AtlasSight DNA Stain (Bioatlas).

### 2.2.5. Sensitivity test of developed multiplex PCR

The sensitivity of the developed multiplex PCR was examined using *Y. enterocolitica* O3 (strain S33), *Y. enterocolitica* O8 (strain YE16-58), *Y. pseudotuberculosis* 1b (strain SP-20), and *Y. pseudotuberculosis* 4b (strain SP-2067). The bacterial cells of each strain from colonies on TSA were suspended in TE buffer to achieve a final concentration of $10^9$ CFU/ml. To examine the detection limits for the developed multiplex PCR, a serial 10-fold dilution of these strains with TE buffer was performed. Genomic DNA from each dilution was obtained using the alkaline-heat DNA extraction method described in Chapter 2.2.2 and was used for multiplex PCR amplification. Aliquots of the serial dilutions were plated in duplicates onto TSA and grown at 25°C for 24 h to determine the number of colony-forming units (CFU).

### 2.2.6. Detection of pathogenic *Yersinia* from spiked rabbit blood samples

Rabbit blood samples artificially inoculated with *Y. enterocolitica* O3 (strain S3-3), *Y. enterocolitica* O8 (strain YE16-58), and *Y. pseudotuberculosis* 1b (strain SP-20) were used. The *Yersinia* strains on TSA were suspended in sterile saline (0.9 % sodium chloride solution) to achieve a final concentration of $10^9$ CFU/mL. A serial 10-fold dilutions of these strains with saline were performed, and the appropriate bacterial dilution was added to defibered rabbit blood (Japan Lamb Co., Ltd., Hiroshima, Japan) to achieve a concentration of $10^0$ to $10^6$ CFU/mL. The number of CFUs was determined by the plate counting method as described in Chapter 2.2.5. Genomic DNA from spiked blood samples (200µL) was prepared using the ISOSPIN Blood & Plasma DNA kit (Nippon Gene Co., Ltd., Tokyo, Japan)

following the manufacturer's instructions, with 100 μL elution buffer added for DNA collection. The collected DNA was used for multiplex PCR amplification.

### 2.2.7. Detection of pathogenic *Yersinia* from wild rodent fecal samples

A total of 45 wild rodent fecal samples, which were captured in in June 2020 (Table 1.1) naturally contaminated with *Y. enterocolitica* O8 were used. The fecal samples (approximately 0.5 g) were suspended in 9 times amount of PBS (pH 7.2), and 200 μl of the PBS suspension was subjected to DNA extraction. Genomic DNA was extracted immediately after the fecal samples were homogenized in PBS without enrichment. It was purified using the QIAamp DNA stool mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions, with 100 μl elution buffer added for DNA collection and used for multiplex PCR amplification. *Y. enterocolitica* O8 was isolated from wild rodent feces using the cold enrichment culture method and was identified as described in Chapter 1.2.2.

## 2.3. RESULTS

### 2.3.1. The specificity of the developed multiplex PCR

The results of the specificity test for the monoplex and multiplex PCR are shown in Table 2.1. Detection of the *fyuA, ail, inv,* and *virF* genes correlated well with the genetic traits of highly pathogenic *Y. enterocolitica*, low pathogenic *Y. enterocolitica,* and *Y.*

*pseudotuberculosis*. Typical examples of multiplex PCR assays for pathogenic *Yersinia* species are shown in Figure 2.1. Among the 25 different pathogenic *Yersinia* strains, only highly pathogenic *Y. enterocolitica* showed an extra PCR product of 253 bp, which corresponded to a part of the *fyuA* gene. The 351 bp fragment of the *ail* gene was observed in all the pathogenic *Y. enterocolitica* serotypes, and the 183 bp fragment of the *inv* gene was detected in all the *Y. pseudotuberculosis* serotypes. The amplicon of 561 bp, which corresponded to a part of the *virF* gene, was observed in all the pathogenic *Yersinia* serotypes tested. The pattern with 2 bands, 253 bp and 351 bp, indicated the presence of a highly pathogenic *Y. enterocolitica* strains. The single-band, 351 bp corresponded to low pathogenic *Y. enterocolitica* strains, and 183 bp corresponded to *Y. pseudotuberculosis* strains. The 561 bp band indicated the presence of a virulent plasmid of the *Yersinia* strains. Thus, the highly pathogenic *Y. enterocolitica*, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis* can be differentiated by 3 different band patterns. No targeted gene products were amplified from the negative controls (Figure 2.1).

### 2.3.2. The sensitivity of the developed multiplex PCR

The results showed that the multiplex PCR was able to detect the pathogenic *Yersinia* with $10^1$–$10^3$ CFU per reaction tube. Among the 4 strains tested, *Y. enterocolitica* O8 (strain YE16-58) and 2 strains of *Y. pseudotuberculosis* 1b (strain SP-20) and 4b (strain SP-2067) were detectable at $10^1$ CFU per reaction tube. However, more than $10^3$ CFU per reaction tube was required to detect *Y. enterocolitica* O3 (strain S3-3) (Figure 2.2).

### 2.3.3. Evaluation of the developed multiplex PCR for detection of pathogenic *Yersinia* from spiked rabbit blood samples

The detection limits of multiplex PCR method for identifying pathogenic *Yersinia* strains in rabbit blood samples were $10^2$–$10^3$ CFU per reaction tube (Figure 2.3). Among the 3 strains tested, *Y. enterocolitica* O8 (strain YE16-58) and *Y. pseudotuberculosis* 1b (strain SP-20) were required only $10^2$ CFU per reaction tube to be detected in artificially inoculated rabbit blood samples. However, more than $10^3$ CFU per reaction tube was required to detect *Y. enterocolitica* O3 (strain S3-3) in artificially inoculated rabbit blood samples. No targeted gene products were amplified from any of blood samples without bateria inoculated.

### 2.3.4. Evaluation of the developed multiplex PCR for detection of pathogenic *Yersinia* from wild rodent fecal samples

The multiplex PCR results were in agreement with those from the culture method (Table 2.3 and Figure 2.4). Among the 45 naturally contaminated wild rodent fecal samples tested, simultaneous amplification of the *virF*, *ail,* and *fyuA* genes was observed in 3 (6.7%) samples, indicating the presence of pathogenic *Y. enterocolitica* O8. These samples were the same as those of the culture-positive samples. No PCR product was observed in the culture-negative samples.

## 2.4. DISCUSSION

A few multiplex PCR methods to detect *Y. enterocolitica* and *Y. pseudotuberculosis* have been reported [Nakajima et al., 1992, Weynants et al., 1996, Kot et al., 2007, Stenkova et al., 2008]. However, no reports are available on the multiplex PCR method for simultaneous detection and identification of highly pathogenic *Y. enterocolitica* and low pathogenic *Y. enterocolitica* at the same time. A rapid, specific, and sensitive multiplex PCR method, which can detect and distinguish the 3 pathogenic *Yersinia* groups consisting of highly pathogenic *Y. enterocolitica*, including serotype O8, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis,* was developed in this study. A new primer pair targeting *fyuA* was designed to detect highly pathogenic *Y. enterocolitica*. This *fyuA* primer pair was combined with the *ail, inv,* and *virF* primer pairs described previously by Thoerner et al. [2003] to allow both detection and differentiation among the 3 pathogenic *Yersinia* groups. The primer pairs, *ail, inv,* and *virF,* were initially designed and used in conventional monoplex PCR assays [Thoerner et al., 2003]. Under the multiplex PCR conditions with a mixture of these 4 pairs of primers (Figure 2.1), 3 groups of pathogenic *Yersinia,* were distinguished. Moreover, the detection limits of the multiplex PCR method were $10^1$–$10^3$ CFU per reaction tube from pure culture of pathogenic *Yersinia* and $10^2$–$10^3$ CFU per reaction tube from rabbit blood samples artificially inoculated with pathogenic *Yersinia*, which demonstrated a high sensitivity level [Weynants et al., 1996, Kot et al., 2007]. A few reports [Jourdan et al., 2000, Bhaduri, 2002] have stated that some primer sets of PCR methods for detecting pathogenic *Yersinia* showed high sensitivity in spiked fecal samples but not in naturally contaminated samples. Therefore, the multiplex PCR method was applied to detect pathogenic *Yersinia* from wild rodent feces naturally contaminated with *Y. enterocolitica* O8 to determine the usefulness of this method as a diagnostic tool. The multiplex PCR method developed in this study could detect *Y.*

*enterocolitica* O8 from the same rodent fecal samples that were culture-positive (Table 2.3, Figure 2.3). While the culture method is time-consuming and laborious [Fukushima et al., 2011, Konishi et al., 2016, Hashimoto et al., 2021], the multiplex PCR method can be completed within 1 day. Therefore, the multiplex PCR developed in this study seems to be a useful method for the rapid and sensitive diagnosis, distinguishing 3 pathogenic *Yersinia* groups such as highly pathogenic *Y. enterocolitica,* including serotype O8, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis*.

## 2.5. SUMMARY

A multiplex PCR method for rapid and sensitive diagnosis, differentiating 3 pathogenic *Yersinia* groups such as the highly pathogenic *Y. enterocolitica*, including serotype O8, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis,* was developed. The multiplex PCR targets 4 genes, *fyuA*, *ail*, *inv*, and *virF,* responsible for the virulence in pathogenic *Yersinia* species. Under the multiplex PCR conditions, the unique band patterns for the highly pathogenic *Y. enterocolitica,* low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis* were generated from *Yersinia* strains, respectively. The detection limits of this method were $10^1$–$10^3$ CFU per reaction tube from pure culture and $10^2$–$10^3$ CFU per reaction tube from spiked rabbit blood samples. This multiplex PCR method could detect pathogenic *Y. enterocolitica* O8 from the wild rodent fecal samples that were culture-positive. Therefore, the new multiplex PCR method developed in this study is a useful tool for rapid and sensitive diagnosis, distinguishing 3 pathogenic *Yersinia* groups.

# CHAPTER 3

## Development of multiplex real-time PCR methods for differentiating highly pathogenic *Yersinia enterocolitica* and low pathogenic *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*

## 3.1. INTRODUCTION

A new multiplex PCR method for diagnosis and distinguishing 3 pathogenic *Yersinia* groups was developed in Chapter 2. Although this method shows relatively rapid and sensitive to detect pathogenic *Yersinia*, it takes 1 day to complete all procedure. Real-time PCR methods are known to be more rapid and sensitive rather than conventional PCR methods [Mackay, 2004, Maurin, 2012]. Some researchers indicated that the real-time PCR methods can be completed for detection of bacteria within 2–3 h [Sen, 2000, Sen and Asher, 2001].

In Chapter 3, we conducted this study to develop multiplex real-time PCR methods such as SYBR Green and TaqMan for detection and differentiation highly pathogenic *Y. enterocolitica*, low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis.* Moreover, the diagnostic performance of the developed SYBR Green and TaqMan multiplex real-time PCR methods was also evaluated.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Bacterial strains

The same set of bacterial strains described in Chapter 2.2.1 was used in this Chapter (Table 2.1 and Table 3.1).

### 3.2.2. DNA extraction from bacteria

The bacterial genomic DNA used for SYBR Green and TaqMan multiplex real-time PCR methods was extracted as described in Chapter 2.2.2.

### 3.2.3. Primers and probes selection

#### 3.2.3.1. SYBR Green real-time PCR primers

Three genes such as *ail*, *fyuA,* and *inv,* which were shown in Chapter 2.2.3, were used as targets for SYBR Green multiplex real-time PCR method. When SYBR Green multiplex real-time PCR method was designed, *virF* gene was not important to differentiate between highly pathogenic *Y. enterocolitica* and low pathogenic one. Therefore, *virF* gene was not used in this multiplex real-time PCR method. Specific SYBR Green real-time PCR primers targeting *fyuA* and *inv* genes were designed in this study using *fyuA* and *inv* gene sequences (GenBank accession no. Z35486.1 and M17448, respectively) by Primer-BLAST software [Ye et al., 2012]. The *ail* primers were designed by Mäde et al. [2008]. The sequences of primers for SYBR Green real-time PCR method are shown in Table 3.2.

#### 3.2.3.2. TaqMan real-time PCR primers and probes

The same primers targeting the *fyuA* and *inv* genes designed for the SYBR Green real-time PCR method were also used in the TaqMan real-time PCR method. The *fyuA* and *inv*

fluorescently labeled oligonucleotide probes were designed for this study. Both *fyuA* and *inv* probes were labeled at the 5' end with reporter dye 6-carboxyflourescein (FAM) and the 3' end with black hole quencher (BHQ1). The primers and probe targeting *ail* gene of *Y. enterocolitica* were designed by Lambertz et al. (2008b); however, the *ail* probe was modified by labeling with Yakima Yellow dye (YAKYE) at the 5' end and Minor Groove Binder Eclipse quencher (MGBEQ) at the 3' end to minimize fluorescence spectrum overlap in this study. The sequences of primers and probes for TaqMan real-time PCR method are shown in Table 3.3.

### 3.2.4. Multiplex real-time PCR method

#### 3.2.4.1. SYBR Green multiplex real-time PCR method

The SYBR Green multiplex real-time PCR master mixture comprised 1X TB Green *Premix* Ex *Taq* II (Tli RNaseH Plus; Takara Bio Inc.), *ail* primers to a final concentration of 0.2 µM, *fyuA* and *inv* primers to a final concentration of 0.15 µM, and 2 µL of template DNA. Sterile distilled water was used to adjust the volume of each reaction mixture to 20 µL. The SYBR Green real-time PCR reaction was performed under the following conditions: initial denaturation step for 30 sec at 95°C, followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec. After PCR amplification, a melting temperature curve analysis was performed on a range from 65°C to 95°C at a rate of 0.5°C per 5 sec. The SYBR Green multiplex real-time PCR assay was carried out in a MiniOpticon Real-Time PCR system (Bio-Rad).

### 3.2.4.2. TaqMan multiplex real-time PCR method

The TaqMan multiplex real-time PCR master mixture comprised 1X *Premix* Ex *Taq* (Perfect Real Time; Takara Bio Inc.), probes to a final concentration of 0.4 μM, *fyuA* and *ail* primers to a final concentration of 0.2 μM, *inv* primers to a final concentration of 0.1 μM, and 2 μL of template DNA. Sterile distilled water was used to adjust the volume of each reaction mixture to 20 μL. The TaqMan multiplex real-time PCR reaction was performed under the following conditions: initial denaturation step for 30 sec at 95°C, followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec. The TaqMan multiplex real-time PCR reactions were performed in the same system as SYBR Green multiplex real-time PCR.

### 3.2.5. Sensitivity test of the developed multiplex real-time PCR methods

Sensitivity tests of the developed SYBR Green and TaqMan multiplex methods were performed according to the protocol described in Chapter 2.2.5. The same pathogenic *Yersinia* strains that were used to determine the sensitivity of the multiplex PCR method in Chapter 2.2.5, were used to examine the sensitivity of the developed SYBR Green and TaqMan multiplex real-time PCR methods.

### 3.2.6. Detection of pathogenic *Yersinia* from spiked rabbit blood

To evaluate the performance of the SYBR Green and TaqMan multiplex methods developed in this study to detect pathogenic *Yersinia* in clinical samples, rabbit blood samples

that were artificially inoculated with pathogenic *Yersinia* strains, were used. These experiments were carried out following the protocol described in Chapter 2.2.6 with the same pathogenic *Yersinia* strains.

### 3.2.7. Detection of pathogenic *Yersinia* from wild rodent fecal samples

The clinical performance of SYBR Green and TaqMan multiplex real-time PCR methods developed in this Chapter was evaluated using the same wild rodent fecal samples that were used in Chapter 2.2.7.

## 3.3. RESULTS

### 3.3.1. The specificity of the developed multiplex real-time PCR

The results of SYBR Green and TaqMan multiplex real-time PCR methods for all bacterial strains are shown in Table 3.1. Detection of the *ail, fyuA,* and *inv* genes corresponded well with the genetic traits of highly pathogenic *Y. enterocolitica*, low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis.* Real-time amplification signals were not generated from any of the negative control strains (Table 3.1).

Typical melting curves obtained from the SYBR Green multiplex real-time PCR method for 3 groups of pathogenic *Yersinia* are illustrated in Figure 3.1. The melting temperatures (Tm) of each target were 78.5°C for *ail* amplicons, 84.5°C for *fyuA* amplicons,

and 81.5°C for *inv* amplicons. Correspondingly, the melting curves demonstrated 2 peaks at Tm 78.5°C and 84.5°C, indicated the present of highly pathogenic *Y. enterocolitica*. The melting curves demonstrated only 1 peak at Tm 78.5°C, corresponded to low pathogenic *Y. enterocolitica* strain, and melting curves demonstrated 1 peak at Tm 81.5°C, corresponded to *Y. pseudotuberculosis* strains. The SYBR Green multiplex real-time PCR method showed a high specificity in the detection of 3 pathogenic *Yersinia* groups.

Typical amplification curves obtained from the TaqMan multiplex real-time PCR method for the highly pathogenic *Y. enterocolitica,* low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis* are illustrated in Figure 3.2. Of the 25 pathogenic *Yersinia* strains tested, all strains successfully demonstrated the unique TaqMan real-time PCR pattern to their pathogenic groups according to the expected amplification patterns (Table 3.1 and Figure 3.2). Therefore, 3 pathogenic *Yersinia* groups can be differentiated by 3 amplification patterns using the TaqMan multiplex real-time PCR (Table 3.1).

### 3.3.2. The sensitivity of the developed multiplex real-time PCR

The results of the SYBR Green and TaqMan multiplex real-time PCR methods showed that all 4 strains tested, including *Y. enterocolitica* O8 (strain YE16-58), *Y. enterocolitica* O3 (strain S3-3), *Y. pseudotuberculosis* 1b (strain SP-20), and *Y. pseudotuberculosis* 4b (strain SP-2067), were detectable at $10^1$ CFU per reaction tube (Table 3.4 and Figure 3.3). As illustrated in Figure 3.3, the Cq value for target gene increased as the concentration of pathogenic *Yersinia* decreased. Standard curves showed that there was a good linear correlation between the Cq values and the concentrations of input bacterial genomic DNA (Figure 3.3). The developed SYBR Green and TaqMan multiplex real-time PCR showed the same sensitivity level.

### 3.3.3. Evaluation of the developed real-time PCR methods for detection of pathogenic *Yersinia* from spiked rabbit blood

The results of the SYBR Green and TaqMan multiplex real-time PCR methods showed that pathogenic *Yersinia* strains artificially inoculated into rabbit blood samples were detectable at $10^1$–$10^2$ CFU per reaction tube (Table 3.5). Among the 3 strains tested, only $10^1$ CFU per reaction tube was required to detect *Y. enterocolitica* O8 (strain YE16-58) and *Y. enterocolitica* O3 (strain S3-3) in artificially inoculated rabbit blood samples. However, more than $10^2$ CFU per reaction tube was required to detect *Y. pseudotuberculosis* 1b (strain SP-20). No amplification was observed in any of the blood samples without bacteria inoculated.

### 3.3.4. Evaluation of the developed multiplex real-time PCR methods for detection of pathogenic *Yersinia* from wild rodent fecal samples

The results of SYBR Green and TaqMan multiplex real-time PCR methods were identical to those of culture method and multiplex PCR method (Table 2.3 and Table 3.6). Among the 45 naturally contaminated wild rodent fecal samples tested, 3 (6.7%) samples were PCR positive for *ail* and *fyuA* genes. This result indicated that these samples were contaminated with pathogenic *Y. enterocolitica* O8. These samples were the same as those of culture-positive and multiplex PCR-positive samples. No PCR amplification was observed in the culture-negative and multiplex PCR-negative samples.

## 3.4. DISCUSSION

A few SYBR Green and TaqMan multiplex real-time PCR methods for the detection of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* have been developed [Fredriksson-Ahomaa et al., 2009, Thomas et al., 2017, Foley et al., 2019]. However, multiplex real-time PCR method for simultaneously detection and differentiation of highly pathogenic *Y. enterocolitica,* low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis* has not been reported. This is the first report of development of SYBR Green and TaqMan multiplex real-time PCR methods for detection and differentiation of 3 pathogenic *Yersinia* groups at the same time. The specificity results obtained with 25 bacterial strains were in agreement with those from the conventional multiplex PCR developed in Chapter 2 (Table 2.1 and Table 3.1). The detection limits of the SYBR Green and TaqMan multiplex real-time methods developed in this study were $10^1$ CFU per reaction tube from pure culture of pathogenic *Yersinia,* and $10^1$–$10^2$ CFU per reaction tube from rabbit blood samples artificially inoculated with pathogenic *Yersinia,* which were comparable to other real-time PCR methods [Lambertz et al., 2008a, 2008b, Thomas et al., 2017]. This sensitivity levels were 10 times higher than those of multiplex PCR method described in Chapter 2. Moreover, the SYBR Green and TaqMan multiplex real-time PCR methods developed in this study could detect *Y. enterocolitica* O8 from the same rodent fecal samples that were culture-positive and conventional multiplex PCR-positive (Table 2.3 and Table 3.6). In addition, the multiplex real-time PCR methods can be completed within 2–3 h although the conventional multiplex PCR method requires 1 day to complete (Chapter 2). These results indicated that the SYBR Green and TaqMan multiplex real-time PCR are more rapid and sensitive compared to the conventional multiplex PCR method, and are useful diagnostic tools for detection of highly pathogenic *Y. enterocolitica*, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis.*

The SYBR Green and TaqMan multiplex real-time PCR methods can be used to provide quantification of the pathogenic *Yersinia* in the sample which the conventional multiplex PCR method does not allow. However, the conventional multiplex PCR method does not require expensive equipment and reagents to perform. Comparing both real-time PCR methods, the SYBR Green multiplex real-time PCR method detects PCR product by SYBR Green dye, which is less expensive than TaqMan probe. Moreover, it is difficult to design the probes for TaqMan multiplex real-time PCR method. Therefore, SYBR Green multiplex real-time PCR method is less expensive and easier to perform compared to the TaqMan multiplex real-time PCR method. However, fluorescent SYBR Green dye binds to all PCR products, including primer-dimer, which may result in a less accurate quantitative analysis [Maurin, 2012]. In contrast, the sequence-specific design oligonucleotide double labeled probe allows accurate quantitative analysis although TaqMan probe is more expensive than SYBR Green [Mackay, 2004, Maurin, 2012]. Therefore, researchers should choose a suitable method based on the objectives of their study.

## 3.5. SUMMARY

The novel SYBR Green and TaqMan multiplex real-time PCR methods for rapid and sensitive diagnosis and differentiating 3 pathogenic *Yersinia* groups were developed in this Chapter. Three virulence genes, including *fyuA*, *ail*, and *inv,* were selected for the development of these multiplex real-time PCR methods. The SYBR Green and TaqMan multiplex real-time PCR methods showed highly specificity for the detection and differentiation of 3 pathogenic *Yersinia* groups. Both SYBR Green and TaqMan multiplex real-time PCR methods showed the similar detection limits ($10^1$ CFU per reaction tube from pure cultures and $10^1$–$10^2$ CFU per reaction tube from spiked rabbit blood samples). This sensitivity is 10 times higher than those of the multiplex PCR method developed in Chapter 2. In addition, the SYBR Green and TaqMan multiplex real-time PCR methods could detect pathogenic *Y. enterocolitica* O8 from wild rodent fecal samples that were culture-positive and conventional multiplex PCR-positive. Both multiplex real-time PCR run take approximately only 2–3 h although the conventional multiplex PCR method required 1 day to detect and distinguish the pathogenic *Yersinia* strains. Therefore, the novel SYBR Green and TaqMan multiplex real-time PCR methods developed in this study are more rapid and sensitive to diagnose and distinguish 3 pathogenic *Yersinia* groups compared to the conventional multiplex PCR method developed in Chapter 2.

# CONCLUSIONS

The main objectives of this present study are to clarify the epidemiological aspects of pathogenic *Yersinia* in wild rodents in Fukushima prefecture, Japan and to develop rapid, sensitive, and specific detection methods for 3 pathogenic *Yersinia* groups such as the highly pathogenic *Y. enterocolitica*, including serotype O8, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis*. The main findings of this study are summarized as follows:

1. From 2012 to 2021, pathogenic *Yersinia enterocolitica* O8 was isolated from 13 (1.7%) of 755 wild rodents captured in Fukushima Prefecture, Japan. All of *Y. enterocolitica* O8 isolates were pathogenic strains, harboring 3 virulent genes, including *ail, fyuA*, and *virF*. Of 13 pathogenic *Y. enterocolitica* O8 positive rodents, 6 (1.2%) were from 464 *A. speciosus*, and 7 (3.0%) were from 232 *A. argenteus*. No *Y. enterocolitica* O8 isolate was recovered from *M. montebelli* and *U. talpoides*. The *Y. enterocolitica* O8 was isolated repeatedly from wild rodents in April 2015, 2016, and 2017, in June and November 2020, and in April 2021, which was 6 of 19 times of observations. The same PFGE patterns were observed from all *Y. enterocolitica* O8 isolates. These results indicated that the same clone of pathogenic *Y. enterocolitica* O8 has been maintained in wild rodent populations in Fukushima Prefecture for at least 6 years. Therefore, wild rodent populations contribute substantially to the persistence of pathogenic *Y. enterocolitica* O8 in the ecosystem. This is the first report on the isolation of pathogenic *Y. enterocolitica* O8 in wild rodents in Fukushima Prefecture, Japan.

2. A multiplex PCR method targeting 4 virulence genes, *fyuA, ail, inv,* and *virF,* of pathogenic *Yersinia* was developed. Under the multiplex PCR conditions, the unique band patterns for the highly pathogenic *Y. enterocolitica,* low pathogenic *Y. enterocolitica,* and *Y. pseudotuberculosis* were generated from *Yersinia* strains. The detection limits of this method were $10^1$–$10^3$ CFU per reaction tube from pure culture, and $10^2$–$10^3$ CFU per reaction tube

from spiked rabbit blood samples. This multiplex PCR method could detect pathogenic *Y. enterocolitica* O8 from the wild rodent fecal samples that were culture-positive. Therefore, the new multiplex PCR method developed in this study is a useful tool for rapid and sensitive diagnosis, distinguishing 3 pathogenic *Yersinia* groups.

3. The novel SYBR Green and TaqMan multiplex real-time PCR methods for rapid and sensitive diagnosis and differentiating 3 pathogenic *Yersinia* groups were developed in this Chapter. Three virulence genes, including *fyuA*, *ail*, and *inv,* were selected for the development of these multiplex real-time PCR methods. The SYBR Green and TaqMan multiplex real-time PCR methods showed highly specificity for the detection and differentiation of 3 pathogenic *Yersinia* groups. Both SYBR Green and TaqMan multiplex real-time PCR methods showed similar detection limits ($10^1$ CFU per reaction tube from pure cultures and $10^1$–$10^2$ CFU per reaction tube from spiked rabbit blood samples). This sensitivity is 10 times higher than those of the multiplex PCR method developed in Chapter 2. In addition, the SYBR Green and TaqMan multiplex real-time PCR methods could detect pathogenic *Y. enterocolitica* O8 from wild rodent fecal samples that were culture-positive and conventional multiplex PCR-positive. Both multiplex real-time PCR run take approximately only 2–3 h although the conventional multiplex PCR method required 1 day to detect and distinguish the pathogenic *Yersinia* strains. Therefore, the novel SYBR Green and TaqMan multiplex real-time PCR methods developed in this study are more rapid and sensitive to diagnose and distinguish 3 pathogenic *Yersinia* groups compared to the conventional multiplex PCR method developed in Chapter 2.

4. In the present study, the prevalence of pathogenic *Yersinia* in wild rodents was observed for 10 years and the pathogenic *Y. enterocolitica* O8 has been found to be maintained at least 6 years within the wild rodent populations in Fukushima Prefecture, Japan. These findings can be useful for understanding the epidemiology and ecology of pathogenic

*Yersinia*, especially the pathogenic *Y. enterocolitica* O8 in Japan. Moreover, 3 molecular methods, including multiplex PCR, SYBR Green and TaqMan multiplex real-time PCR, were developed for rapid and sensitive diagnosis, differentiating 3 pathogenic *Yersinia* groups such as the highly pathogenic *Y. enterocolitica*, including serotype O8, low pathogenic *Y. enterocolitica*, and *Y. pseudotuberculosis*. These methods can be valuable tools for clarifying the ecology and epidemiological of pathogenic *Yersinia* in nature and for the diagnosis of *Yersinia* infection in humans.

**AKNOWLEDGMENTS**

I would like to express my heartfelt gratitude to Associate Professor Dr. Hideki HAYASHIDANI, Cooperative Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Japan for his instructions, stimulus, support and valuable advice and criticisms through the experiments and in preparation of this thesis.

I would like to be grateful to Associate Professor Dr. Takahide TANIGUCHI, Tokyo University of Agriculture and Technology, Japan, Professor Dr. Jun TERAJIMA and Professor Dr. Takehisa YAMAMOTO, Iwate University, Japan, and Dr. Yukiko HARA-KUDO, Division of Microbiology, National Institute of Health Sciences, Japan, for their valuable suggestions and critical reading of this manuscript.

I wish to express my sincere gratitude to Associate Professor Dr. HO Trung Thong and my colleagues, Faculty of Animal Science and Veterinary Medicine, Hue University of Agriculture and Forestry, Hue University, Vietnam, for their support, and encouragement.

I sincerely appreciate the great support of Dr. Yukiko SASSA-O'BRIEN, Dr. NGUYEN Khanh Thuan, Dr. TRAN Thi Hong To, Mr. Shunsuke IKEUCHI and other students in Division of Animal Life Science, Institute of Agriculture, Tokyo University of Agriculture and Technology, Japan for their great support to my experiments.

My studying here was supported by the Monbukagakusho scholarship. I would like to thank the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan for that.

Finally, I dedicate this thesis to my family and my parents who always support and give me unconditional love.

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## TABLES AND FIGURES

**Table 1.1.** Prevalence of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* in wild rodents in Fukushima prefecture, Japan by year

| Survey time | | No. of *Yersinia* positive animals/No. of animals examined (%) | | | | |
|---|---|---|---|---|---|---|
| | | *Apodemus speciosus* | *Apodemus argenteus* | *Microtus montebelli* | *Urotrichus talpoides* | Total |
| 2012 | Jul. | 0/9 (0.0) | 0/9 (0.0) | 0 | 0 | 0/18 (0.0) |
| | Nov. | 0/9 (0.0) | 0/10 (0.0) | 0 | 0 | 0/19 (0.0) |
| 2013 | Apr. | 0/27 (0.0) | 0/16 (0.0) | 0 | 0 | 0/43 (0.0) |
| | Nov. | 1/35 (2.9)* | 0/8 (0.0) | 0/7 (0.0) | 0 | 1/50 (2.0)* |
| 2014 | Apr. | 0/28 (0.0) | 0/19 (0.0) | 0/3 (0.0) | 0/7 (0.0) | 0/57 (0.0) |
| | Nov. | 0/21 (0.0) | 0/11 (0.0) | 0 | 0/2 (0.0) | 0/34 (0.0) |
| 2015 | Apr. | 0/61 (0.0) | 3/35 (8.6)§ | 0/3 (0.0) | 0/1 (0.0) | 3/100 (3.0)§ |
| | Nov. | 0/36 (0.0) | 0/4 (0.0) | 0 | 0 | 0/40 (0.0) |
| 2016 | Apr. | 2/38 (5.3)§ | 1/18 (5.6)§ | 0/4 (0.0) | 0/2 (0.0) | 3/62 (4.8)§ |
| | Nov. | 0/17 (0.0) | 0/13 (0.0) | 0 | 0 | 0/30 (0.0) |
| 2017 | Apr. | 0/9 (0.0) | 1/4 (25.0)§ | 0 | 0/4 (0.0) | 1 /17 (5.9)§ |
| | Nov. | 0/12 (0.0) | 0/2 (0.0) | 0 | 0 | 0/14 (0.0) |
| 2018 | Apr. | 0/72 (0.0) | 0/16 (0.0) | 0/17 (0.0) | 0/1 (0.0) | 0/106 (0.0) |
| | Nov. | 0/11 (0.0) | 0/2 (0.0) | 0/2 (0.0) | 0/1 (0.0) | 0/16 (0.0) |
| 2019 | Apr. | 0/12 (0.0) | 0/3 (0.0) | 0 | 0 | 0/15 (0.0) |
| | Nov. | 0/15 (0.0) | 0/6 (0.0) | 0 | 0 | 0/21 (0.0) |
| 2020 | Jun. | 1/20 (5.0)§ | 2/23 (8.7)§ | 0/1 (0.0) | 0/1 (0.0) | 3/45 (6.7)§ |
| | Nov. | 1/25 (4.0)§ | 0/23 (0.0) | 0 | 0/3 (0.0) | 1/51 (2.0)§ |
| 2021 | Apr. | 2/7 (28.6)§ | 0/10 (0.0) | 0 | 0 | 2/17 (11.8)§ |
| Total | | 7/464 (1.5) | 7/232 (3.0) | 0/37 (0.0) | 0/22 (0.0) | 14/755 (1.9) |

* *Y. pseudotuberculosis* O5b

§ *Y. enterocolitica* O8

**Table 1.2.** Prevalence of pathogenic *Yersinia enterocolitica* O8 by sampling area and wild rodent species

| Rodent species | Sex/age | | No. positive animals/No. animals examined (%) | | |
|---|---|---|---|---|---|
| | | | Mt. Kuchibuto | Mt. Hayama | Total |
| *Apodemus speciosus* | Male | Juvenile | 2/59 (3.4) | 0/57 (0.0) | 2/116 (1.7) |
| | | Adult | 0/51 (0.0) | 1/78 (1.3) | 1/129 (0.8) |
| | | Subtotal | 2/110 (1.8) | 1/135 (0.7) | 3/245 (1.2) |
| | Female | Juvenile | 0/45 (0.0) | 1/71 (1.4) | 1/116 (0.9) |
| | | Adult | 2/41 (4.9) | 0/62 (0.0) | 2/103 (1.9) |
| | | Subtotal | 2/86 (2.3) | 1/133 (0.8) | 3/219 (1.4) |
| Subtotal | | | 4/196 (2.0) | 2/268 (0.7) | 6/464 (1.2) |
| *Apodemus argenteus* | Male | | 3/74 (4.1) | 1/40 (2.5) | 4/114 (3.5) |
| | Female | | 3/75 (4.0) | 0/43 (0.0) | 3/118 (2.5) |
| Subtotal | | | 6/149 (4.0) | 1/83 (1.2) | 7/232 (3.0) |
| Total | | | 10/345 (2.9) | 3/351 (0.9) | 13/696 (1.9) |

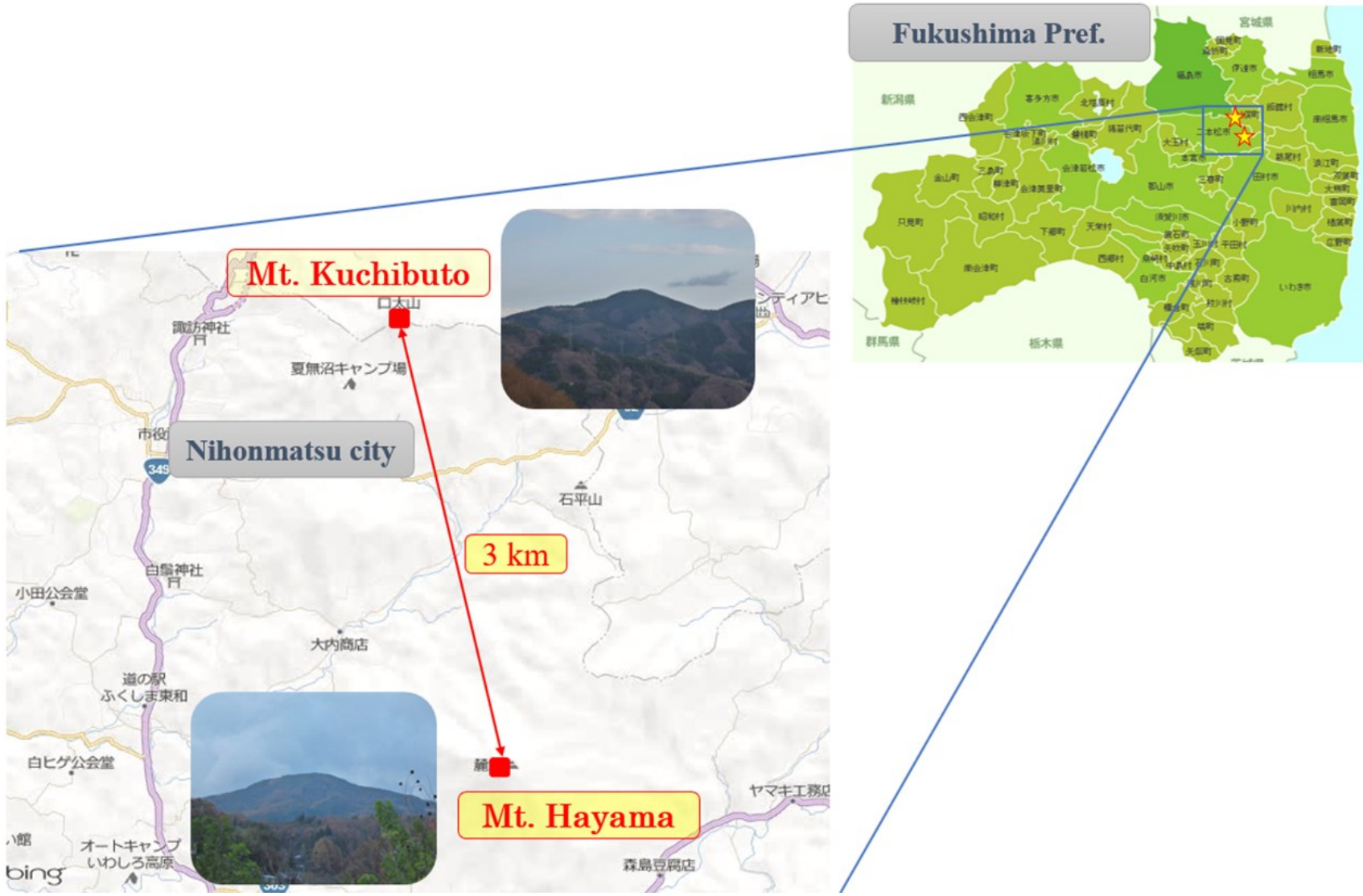


**Figure 1.1.** Map of sampling points, Mt. Kuchibuto and Mt. Hayama in Nihonmatsu city, Fukushima Prefecture, Japan.

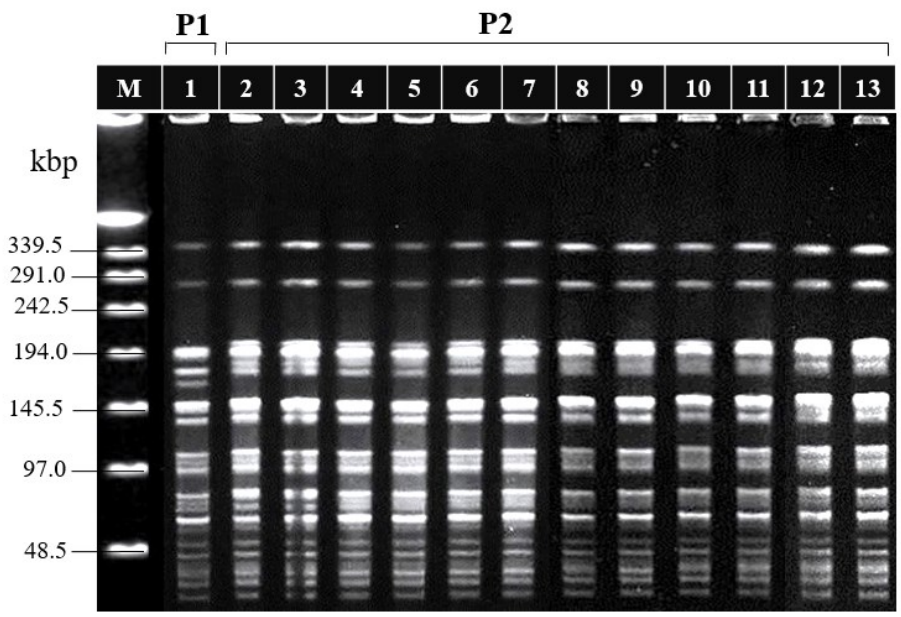


**Figure 1.2.** Pulsed-field gel electrophoresis (PFGE) patterns obtained from 13 *Yersinia enterocolitica* O8 isolates originated from wild rodents in Fukushima Prefecture, Japan by *Not*I enzyme. Lane M, CHEF DNA Size Standard Lambda Ladder. Lane 1 shows PFGE pattern P1 produced from YE15-29 strain isolated in 2015. Lanes 2 to 13 show PFGE pattern P2 produced from 12 strains isolated in 2015, 2016, 2017, 2020, and 2021: lanes 2-3 are isolates in 2015, coded YE15-61, and YE15-65, respectively; lanes 4-6 are isolates in 2016, coded YE16-07, YE16-14, and YE16-58, respectively; lane 7 is isolate in 2017, coded YE17-08; lanes 8-11 are isolates in 2020, coded YE20-06, YE20-07, YE20-23, and YE20-44, respectively; and lanes 12-13 are isolates in 2021, coded YE21-4 and YE21-17, respectively.

**Table 2.1.** Bacteria strains and band patterns of each bacteria by the polymerase chain reaction method (PCR)

| Species | Biotype | Serotype | Strain | PCR results | | | |
|---|---|---|---|---|---|---|---|
| | | | | *inv* | *fyuA* | *ail* | *virF* |
| *Yersinia enterocolitica* | 4 | O3 | S3-3 | − | − | + | + |
| *Y. enterocolitica* | 4 | O3 | S3-8 | − | − | + | + |
| *Y. enterocolitica* | 1B | O4,32 | IP96 | − | + | + | + |
| *Y. enterocolitica* | 2 | O5,27 | S5-250 | − | − | + | + |
| *Y. enterocolitica* | 2 | O5,27 | S5-203 | − | − | + | + |
| *Y. enterocolitica* | 1B | O8 | Ye16-58 | − | + | + | + |
| *Y. enterocolitica* | 1B | O8 | NY9306089 | − | + | + | + |
| *Y. enterocolitica* | 1B | O8 | IP843 | − | + | + | + |
| *Y. enterocolitica* | 2 | O9 | S9-87 | − | − | + | + |
| *Y. enterocolitica* | 2 | O9 | Pa117 | − | − | + | + |
| *Y. enterocolitica* | 1B | O13a,13b | WA285 | − | + | + | + |
| *Y. enterocolitica* | 1B | O13a,13b | WAT568 | − | + | + | + |
| *Y. enterocolitica* | 1B | O18,13b | IP896 | − | + | + | + |
| *Y. enterocolitica* | 1B | O20 | IP1106 | − | + | + | + |
| *Y. enterocolitica* | 1B | O21 | IP1110 | − | + | + | + |
| *Y. pseudotuberculosis* | | 1b | SP-20 | + | − | − | + |
| *Y. pseudotuberculosis* | | 1b | SP-1526 | + | − | − | + |
| *Y. pseudotuberculosis* | | 2b | 1608 | + | − | − | + |
| *Y. pseudotuberculosis* | | 3 | SP-148 | + | − | − | + |
| *Y. pseudotuberculosis* | | 3 | SP-1726 | + | − | − | + |
| *Y. pseudotuberculosis* | | 4b | SP-2067 | + | − | − | + |
| *Y. pseudotuberculosis* | | 4b | SP-2118 | + | − | − | + |
| *Y. pseudotuberculosis* | | 5a | SP-328 | + | − | − | + |
| *Y. pseudotuberculosis* | | 5a | SP-334 | + | − | − | + |
| *Y. pseudotuberculosis* | | 6 | SP-901 | + | − | − | + |
| *Y. enterocolitica* | 1A | O8,19 | NY8904001 | − | − | − | − |
| *Y. aldovae* | | | JCM 5892 | − | − | − | − |
| *Y. intermedia* | | | JCM 7579 | − | − | − | − |
| *Y. kristensenii* | | | JCM 7576 | − | − | − | − |
| *Y. rohdei* | | | JCM 7376 | − | − | − | − |
| *Salmonella enterica* subsp. *enterica* serovar Enteritidis | | | NS9506003 | − | − | − | − |
| *Escherichia coli* | | | JCM 5491 | − | − | − | − |

+, PCR product of expected size; −, no PCR product

**Table 2.2.** Oligonucleotide primers used in conventional PCR method

| Target genes | | Sequences (5'-3') | Product length (bp) | Target pathogens | References |
|---|---|---|---|---|---|
| *inv* | F | CGGTACGGCTCAAGTTAATCTG | 183 | Pathogenic *Yersinia pseudotuberculosis* | Thoerner et al., [2003] |
| | R | CCGTTCTCCAATGTACGTATCC | | | |
| *fyuA* | F | GGCCGTAAGCTCTCACTT | 253 | Highly pathogenic *Y. enterocolitica* (American strains) | This study |
| | R | ACACCATATCAACGGTACGC | | | |
| *ail* | F | TAATGTGTACGCTGCGAG | 351 | Pathogenic *Y. enterocolitica* | Thoerner et al., [2003] |
| | R | GACGTCTTACTTGCACTG | | | |
| *virF* | F | GGCAGAACAGCAGTCAGACATA | 561 | Pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* | Thoerner et al., [2003] |
| | R | GGTGAGCATAGAGAATACGTCG | | | |

F, forward primer; R, reverse primer

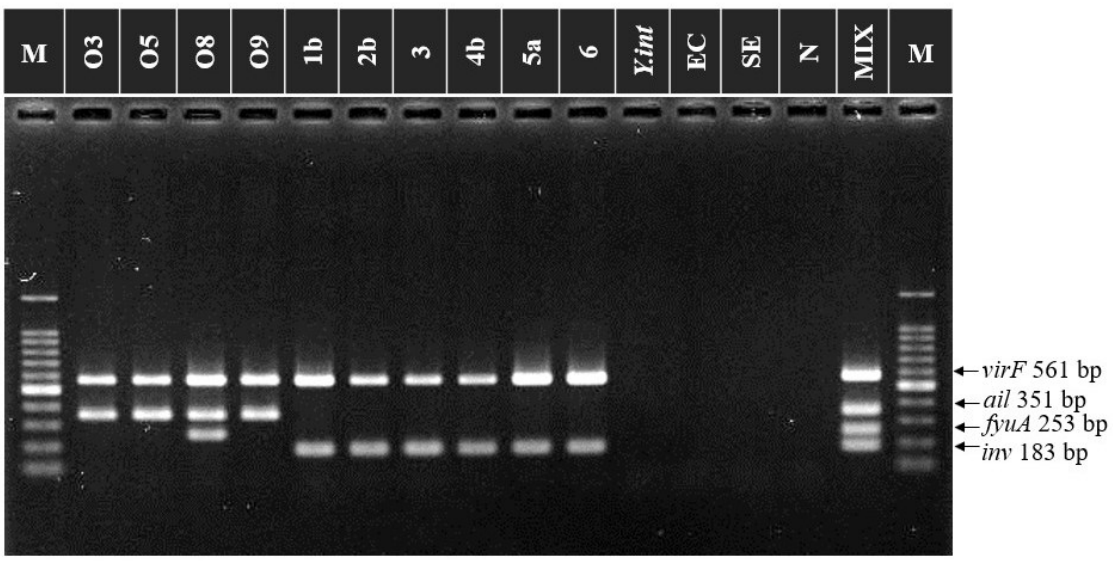


**Figure 2.1.** Agarose gel electrophoresis results for the developed multiplex PCR method with representative isolates of pathogenic *Yersinia* serotypes. Lanes O3, O5, O8, and O9, *Y. enterocolitica* serotype O3, O5,27, O8, and O9, respectively; lanes 1b, 2b, 3, 4b, 5a, and 6, *Y. pseudotuberculosis* serotype 1b, 2b, 3, 4b, 5a and 6, respectively; lane Y.int., *Y. intermedia;* lane EC*, Escherichia coli*; lane SE, *Salmonella enterica* subsp*. enterica* serovar Enteritidis. Lane N, multiplex PCR in the absence of template DNA. Lane MIX, Mix DNA of *Y. enterocolitica* O8 and *Y. pseudotuberculosis* 1b. Lane M, 100 bp DNA ladder (Takara Bio Inc., Kusatsu, Japan).

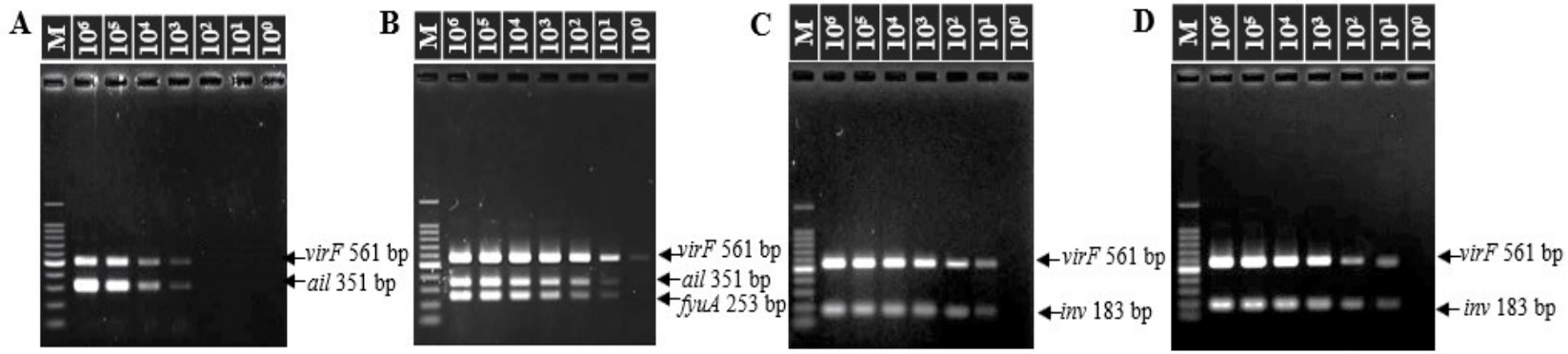


**Figure 2.2.** Detection limits of the multiplex PCR for pure culture of pathogenic *Yersinia*. The numbers above each lane represent $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, $10^1$, and $10^0$ CFU per reaction tube of template DNA of low pathogenic *Y. enterocolitica* O3 (strain S3-3) (A); highly pathogenic *Y. enterocolitica* O8 (strain YE16-58) (B); *Y. pseudotuberculosis* 1b (strain SP-20) (C); and *Y. pseudotuberculosis* 4b (strain SP-2067) (D). Lane M, 100 bp DNA ladder (Takara Bio Inc., Kusatsu, Japan).

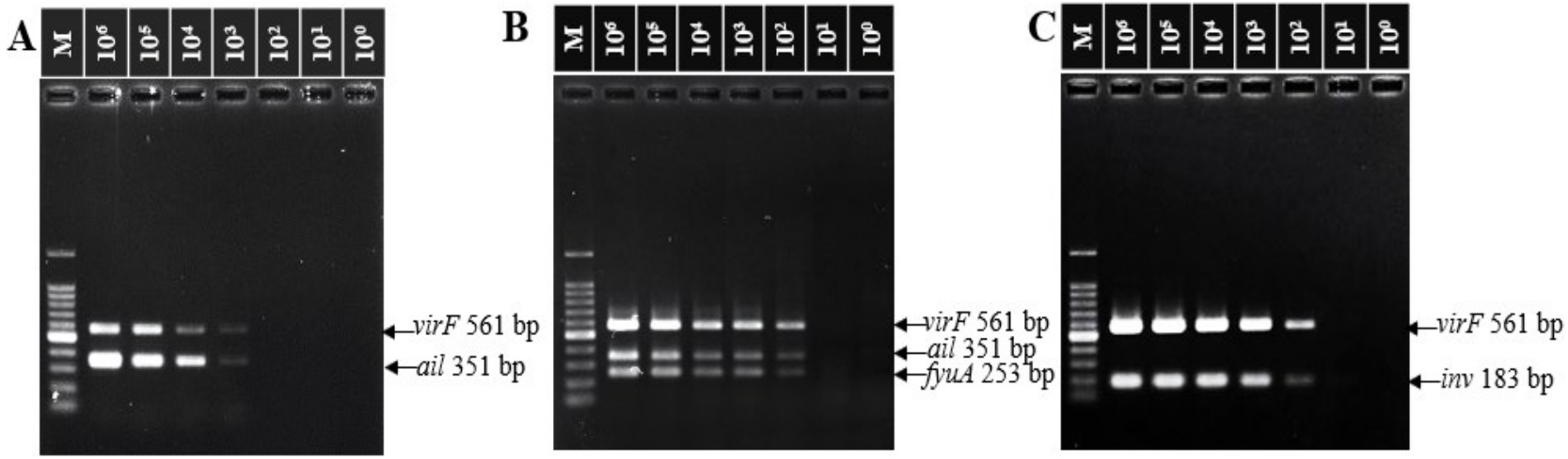


**Figure 2.3.** Detection limits of the multiplex PCR for pathogenic *Yersinia* artificially contaminated rabbit blood samples. The numbers above each lane represent $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, $10^1$, and $10^0$ CFU per reaction tube of template DNA of rabbit blood samples artificially contaminated with low pathogenic *Y. enterocolitica* O3 (strain S3-3) (A); highly pathogenic *Y. enterocolitica* O8 (strain YE16-58) (B); *Y. pseudotuberculosis* 1b (strain SP-20) (C). Lane M, 100 bp DNA ladder (Takara Bio Inc., Kusatsu, Japan).

**Table 2.3.** Comparison of multiplex PCR method with culture method in detecting pathogenic *Yersinia* from wild rodent fecal samples

| Methods | No. of samples | *Y. enterocolitica* | *Y. pseudo-tuberculosis* |
|---|---|---|---|
| Multiplex PCR | 45 | 3 (6.7%)§ | 0 |
| Culture | | 3 (6.7%)§ | 0 |

§*Y. enterocolitica* O8

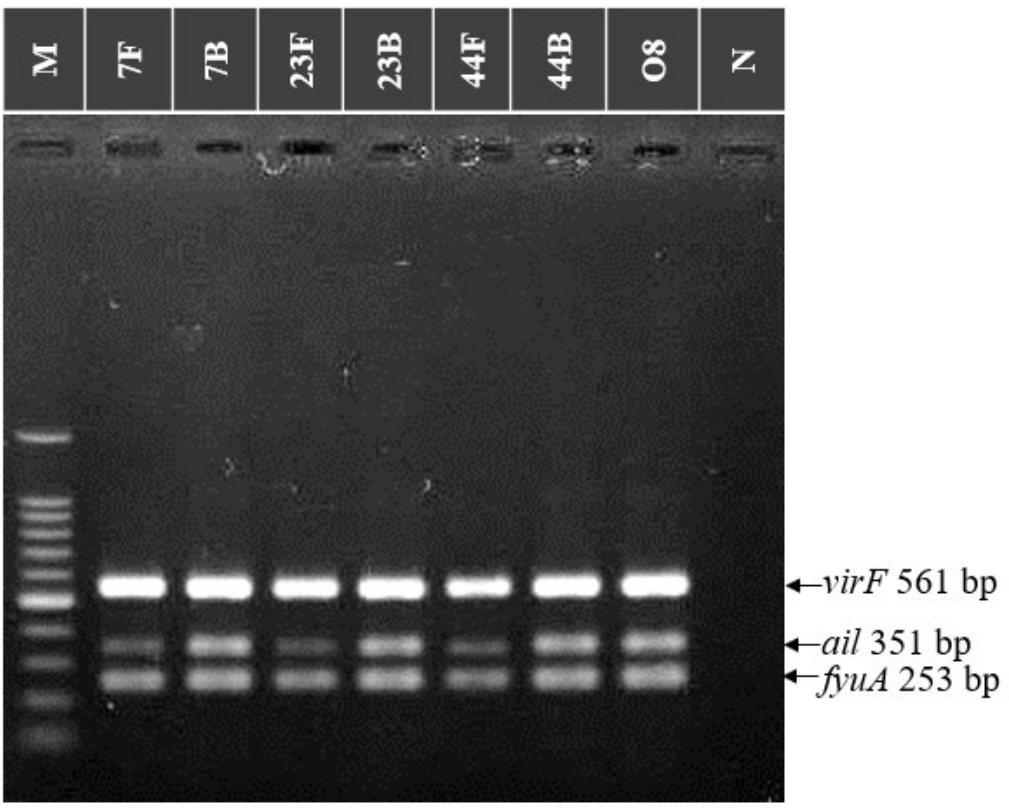


**Figure 2.4.** Band pattern of the developed multiplex PCR for *Yersinia* from fecal samples and *Yersinia* isolates from wild rodents. Lane M, 100 bp DNA ladder (Takara Bio Inc., Kusatsu, Japan). Lanes 7F, 23F, 44F, mice fecal samples number 7, 23, and 44, respectively; 7B, 23B, 44B, *Yersinia* isolates from mice fecal samples number 7, 23, and 44, respectively. Lane O8, positive control - *Y. enterocolitica* O8. Lane N, multiplex PCR in the absence of template DNA.

**Table 3.1.** Bacteria strains used in the present study and results by multiplex real-time PCR

| Species | Biotype | Serotype | Strain | Real-time PCR results | | | Multiplex real-time PCR pattern |
|---|---|---|---|---|---|---|---|
| | | | | *inv* | *fyuA* | *ail* | |
| *Y. enterocolitica* | 4 | O3 | S3-3 | − | − | + | I |
| *Y. enterocolitica* | 4 | O3 | S3-8 | − | − | + | I |
| *Y. enterocolitica* | 1B | O4,32 | IP96 | − | + | + | II |
| *Y. enterocolitica* | 2 | O5,27 | S5-250 | − | − | + | I |
| *Y. enterocolitica* | 2 | O5,27 | S5-203 | − | − | + | I |
| *Y. enterocolitica* | 1B | O8 | Ye16-58 | − | + | + | II |
| *Y. enterocolitica* | 1B | O8 | NY9306089 | − | + | + | II |
| *Y. enterocolitica* | 1B | O8 | IP843 | − | + | + | II |
| *Y. enterocolitica* | 2 | O9 | S9-87 | − | − | + | I |
| *Y. enterocolitica* | 2 | O9 | Pa117 | − | − | + | I |
| *Y. enterocolitica* | 1B | O13a,13b | WA285 | − | + | + | II |
| *Y. enterocolitica* | 1B | O13a,13b | WAT568 | − | + | + | II |
| *Y. enterocolitica* | 1B | O18,13b | IP896 | − | + | + | II |
| *Y. enterocolitica* | 1B | O20 | IP1106 | − | + | + | II |
| *Y. enterocolitica* | 1B | O21 | IP1110 | − | + | + | II |
| *Y. pseudotuberculosis* | | 1b | SP-20 | + | − | − | III |
| *Y. pseudotuberculosis* | | 1b | SP-1526 | + | − | − | III |
| *Y. pseudotuberculosis* | | 2b | 1608 | + | − | − | III |
| *Y. pseudotuberculosis* | | 3 | SP-148 | + | − | − | III |
| *Y. pseudotuberculosis* | | 3 | SP-1726 | + | − | − | III |
| *Y. pseudotuberculosis* | | 4b | SP-2067 | + | − | − | III |
| *Y. pseudotuberculosis* | | 4b | SP-2118 | + | − | − | III |
| *Y. pseudotuberculosis* | | 5a | SP-328 | + | − | − | III |
| *Y. pseudotuberculosis* | | 5a | SP-334 | + | − | − | III |
| *Y. pseudotuberculosis* | | 6 | SP-901 | + | − | − | III |
| *Y. enterocolitica* | 1A | O8,19 | NY8904001 | − | − | − | − |
| *Y. aldovae* | | | JCM 5892 | − | − | − | − |
| *Y. intermedia* | | | JCM 7579 | − | − | − | − |
| *Y. kristensenii* | | | JCM 7576 | − | − | − | − |
| *Y. rohdei* | | | JCM 7376 | − | − | − | − |
| *Salmonella enterica* subsp. *enterica* serovar Enteritidis | | | NS9506003 | − | − | − | − |
| *Escherichia coli* | | | JCM 5491 | − | − | − | − |

+, PCR product of expected Tm value or fluorescent signal; −, no PCR product

**Table 3.2.** Oligonucleotide primers used in SYBR Green real-time PCR method

| Target genes | | Sequences (5'-3') | Product length (bp) | Target pathogens | References |
|---|---|---|---|---|---|
| *ail* | F | GGTTATGCACAAAGCCATGTAAA | 117 | Pathogenic *Yersinia enterocolitica* | Mäde et al., [2008] |
| | R | AAACGAACCTATTACTCCCCAGTT | | | |
| *fyuA* | F | GGCCGTAAGCTCTCACTT | 97 | Highly pathogenic *Y. enterocolitica* (American strains) | This study |
| | R | AGTCATCGGTGGTGTATTTC | | | |
| *inv* | F | GCTTTTGACACAACCTTAGGCAATA | 89 | Pathogenic *Y. pseudotuberculosis* | This study |
| | R | CCCAACGTGGTACTGGTCAATG | | | |

F, forward primer; R, reverse primer

**Table 3.3.** Oligonucleotide primers and probes used in TaqMan real-time PCR method

| Target genes | | Sequences (5'-3') | Product length (bp) | Target pathogens | References |
|---|---|---|---|---|---|
| *ail* | F | ATGATAACTGGGGAGTAATAGGTTCG | 163 | Pathogenic *Yersinia enterocolitica* | Lambert et al., [2008b] |
| | R | CCCAGTAATCCATAAAGGCTAACATAT | | | |
| | P | [YAKYE]TCTATGGCAGTAATAAGTTTGGTCACGGTGATCT[MGBEQ]# | | | |
| *fyuA* | F | GGCCGTAAGCTCTCACTT | 97 | Highly pathogenic *Y. enterocolitica* (American strains) | This study |
| | R | AGTCATCGGTGGTGTATTTC | | | |
| | P | [FAM]CGCTGCACTGACAGCCAGACCCT[BHQ1] | | | |
| *inv* | F | GCTTTTGACACAACCTTAGGCAATA | 89 | Pathogenic *Y. pseudotuberculosis* | This study |
| | R | CCCAACGTGGTACTGGTCAATG | | | |
| | P | [FAM]TGGGCGTTATCACGGATCACAATGAC[BHQ1] | | | |

F, forward primer; R, reverse primer; P, probe

FAM, dye 6-carboxyflourescein; BHQ1, black hole quencher 1; YAKYE, Yakima Yellow dye; MGBEQ, Minor Groove Binder Eclipse quencher

# The reporter dye and quencher dye of this probe were changed to minimize fluorescence spectrum overlap in our assay

**Table 3.4.** Detection limits of the developed SYBR Green and TaqMan multiplex real-time PCR for pure culture of pathogenic *Yersinia*

| *Yersinia* species | CFU per reaction tube | | | | | | |
|---|---|---|---|---|---|---|---|
| | $10^6$ | $10^5$ | $10^4$ | $10^3$ | $10^2$ | $10^1$ | $10^0$ |
| *Y. enterocolitica* O3 | + | + | + | + | + | + | − |
| *Y. enterocolitica* O8 | + | + | + | + | + | + | − |
| *Y. pseudotuberculosis* 1b | + | + | + | + | + | + | − |
| *Y. pseudotuberculosis* 4b | + | + | + | + | + | + | − |

+, PCR product of expected Tm value or fluorescent signal; −, no PCR product

**Table 3.5.** Detection limits of the developed SYBR Green and TaqMan multiplex real-time PCR for pathogenic *Yersinia* from artificially contaminated rabbit blood samples

| *Yersinia* species | CFU per reaction tube | | | | | | |
|---|---|---|---|---|---|---|---|
| | $10^6$ | $10^5$ | $10^4$ | $10^3$ | $10^2$ | $10^1$ | $10^0$ |
| *Y. enterocolitica* O3 | + | + | + | + | + | + | − |
| *Y. enterocolitica* O8 | + | + | + | + | + | + | − |
| *Y. pseudotuberculosis* 1b | + | + | + | + | + | − | − |

+, PCR product of expected Tm value or fluorescent signal; −, no PCR product

**Table 3.6.** Comparison of real-time PCR methods with culture method in detecting pathogenic *Yersinia* from wild rodent fecal samples

| Methods | No. of samples | *Y. enterocolitica* | *Y. pseudo-tuberculosis* |
|---|---|---|---|
| SYBR Green multiplex real-time PCR | 45 | 3 (6.7%)§ | 0 |
| TaqMan multiplex real-time PCR | | 3 (6.7%)§ | 0 |
| Culture | | 3 (6.7%)§ | 0 |

§*Y. enterocolitica* O8

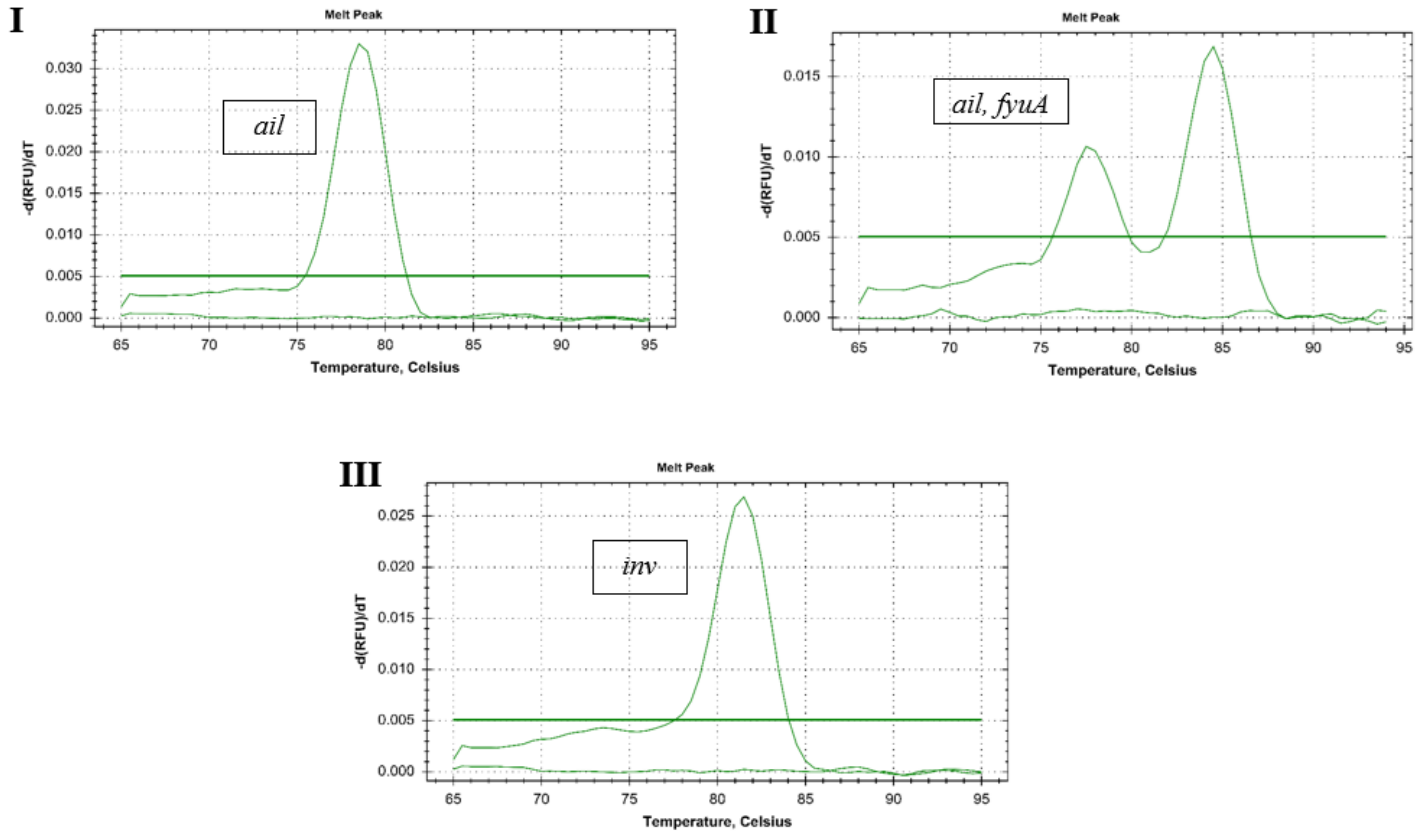


**Figure 3.1.** Melting curve analysis obtained from SYBR Green real-time PCR for low pathogenic *Yersinia enterocolitica* O3 (strain S3-3) (I), highly pathogenic *Y. enterocolitica* O8 (strain YE16-58) (II), and *Y.*

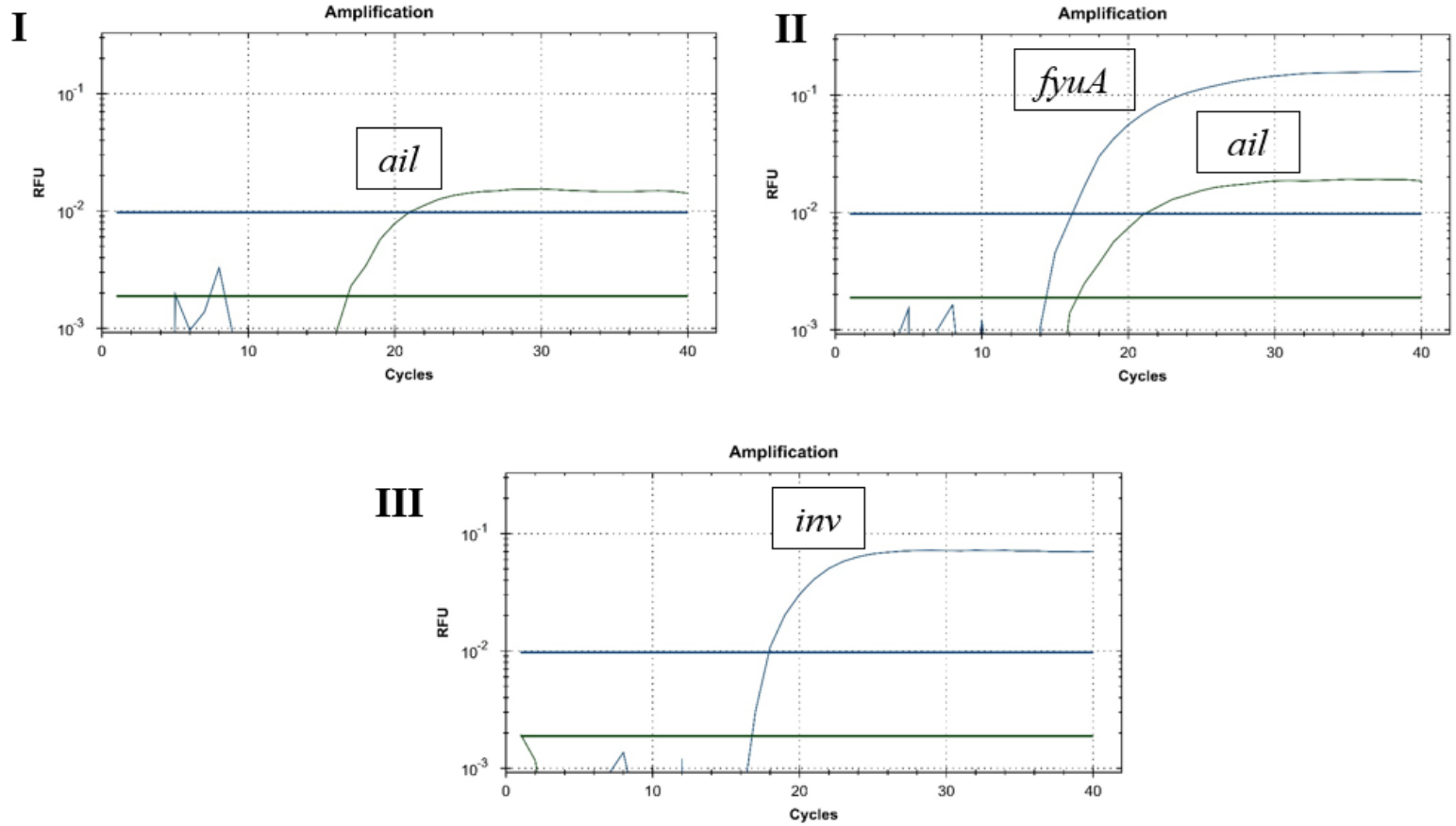


**Figure 3.2.** Amplification curves obtained from TaqMan multiplex real-time PCR for low pathogenic *Yersinia enterocolitica* O3 (strain S3-3) (I), highly pathogenic *Y. enterocolitica* O8 (strain YE16-58) (II), and *Y.*

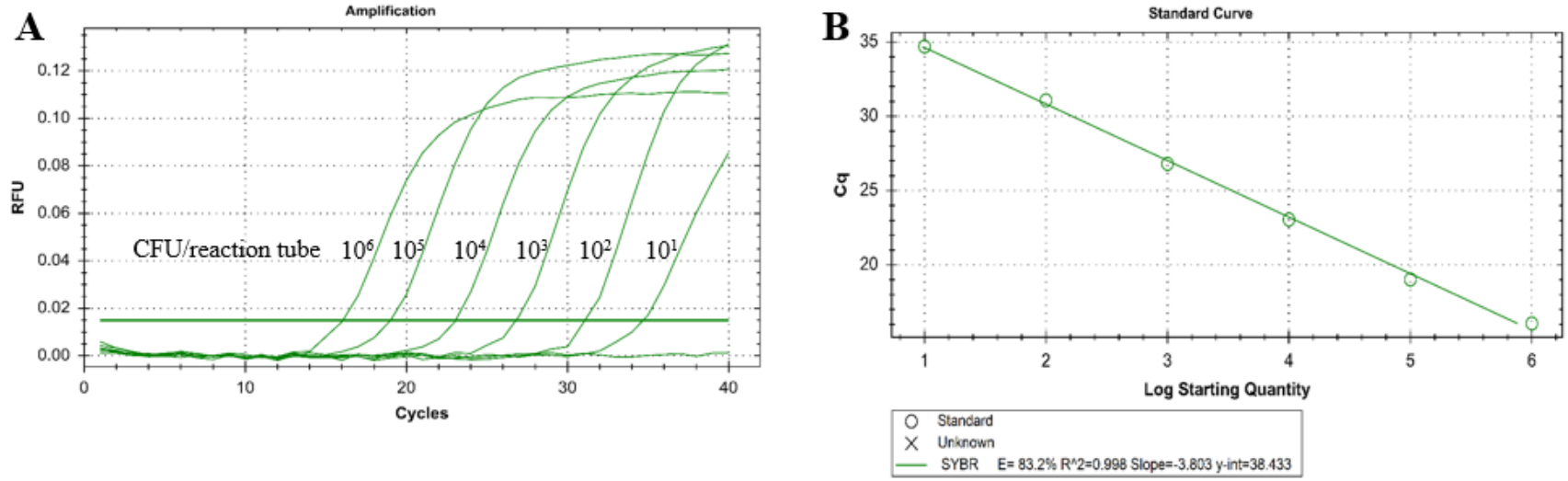


**Figure 3.3.** Detection limit of the developed SYBR Green multiplex real-time PCR.

(A) Amplification curves of the 10-fold serial dilutions of *Yersinia enterocolitica* O8 genomic DNA, (B) Standard curves of the developed SYBR Green multiplex real-time PCR showing amplification of successive 10-fold serial dilutions of *Y. enterocolitica* O8 genomic DNA (from the left $10^1$ – $10^6$ CFU per reaction tube).