Detection and prevalence of pathogenic *Yersinia enterocolitica* in refrigerated and frozen dairy products by duplex PCR and dot hybridization targeting the *virF* and *ail* genes

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ABSTRACT

Pathogenic *Yersinia enterocolitica* is involved in yersiniosis through expression of chromosome-borne or plasmid-borne virulence factors. *Yersinia enterocolitica* is a cold-tolerant pathogen frequently isolated from refrigerated or frozen foods. However, little attention has been focused on the prevalence of pathogenic *Y. enterocolitica* in refrigerated or frozen dairy samples in China. In this study, we developed a new duplex PCR targeting the plasmid-borne *virF* gene and chromosome-borne *ail* gene for detection of pathogenic *Y. enterocolitica* isolates. We established a detection limit for the duplex PCR of 6.5×10^2 cfu/mL in artificially contaminated dairy samples. In addition, the duplex PCR could detect directly 4.5 to 5.7 cfu of *Y. enterocolitica* in 5 mL of brain heart infusion broth after 6 h of enrichment at 28°C. A newly developed dot hybridization assay further confirmed specificity of the duplex PCR for detection of virulent *Y. enterocolitica*. Furthermore, 13 *Y. enterocolitica* and 5 pathogenic strains, from 88 commercial frozen or refrigerated dairy products, were detected successfully by the China National Standard method (GB/T4789.8-2008) and the duplex PCR, respectively. Finally, biotypes and serotypes of pathogenic *Y. enterocolitica* strains were further characterized. The duplex PCR developed here is reliable for large-scale screening, routine monitoring, and risk assessment of pathogenic *Y. enterocolitica* in refrigerated or frozen dairy products.

Key words: pathogenic *Yersinia enterocolitica*, refrigerated or frozen dairy products, duplex PCR, dot hybridization

INTRODUCTION

Yersinia enterocolitica is a common foodborne pathogen causing acute gastroenteritis, enterocolitis, and mesenteric lymphadenitis as well as extraintestinal disorders in humans (Yucel and Ulusoy, 2006; Hanifian and Khani, 2012); it is ubiquitous in animals, foods, and environments (Fredriksson-Ahomaa and Korkeala, 2003). In Europe, yersiniosis is the third most important foodborne enteric infection after campylobacteriosis and salmonellosis (ESFA-ECDC, 2012; Tan et al., 2014). The first human yersiniosis was reported in Malaysia, where *Y. enterocolitica* O:3 was isolated from a 34-yr-old female (Jegathesan et al., 1984). Six biotypes of *Y. enterocolitica* (biotypes 1A, 1B, 2, 3, 4, 5) are known, and only biotype 1A strains are generally considered avirulent (Grant et al., 1998). The biovar/serovar combination most often involved in human disease in Europe is 4/O:3, followed by 2/O:5, 27, 3/O:5, 27 (Gürtler et al., 2005). Nonpathogenic strains are frequently isolated from food samples, and it is therefore essential to distinguish truly pathogenic strains from apathogenic strains. The identification of pathogenic *Y. enterocolitica* is traditionally based on phenotypic tests such as calcium-dependent growth at 37°C, Congo red binding (Prpic et al., 1983), pyrazinamidase testing (Kandolo and Wauters, 1985), autoagglutination testing, and serum-resistance testing (Bhaduri and Cottrell, 1997). However, serological and biochemical classifications are time consuming and have low specificity for pathogenic isolates of *Y. enterocolitica* (Thoerner et al., 2003).

The pathogenicity of *Y. enterocolitica* depends on expression of plasmid and chromosomal determinants (Kuhnert et al., 1997). The *Y. enterocolitica* attachment invasion locus gene (*ail*) is reported to be present in only pathogenic strains (Miller and Falkow, 1988; Miller et al., 1989). Plasmid-borne virulence factors include the *virF* gene, which encodes transcriptional activators of *Y. enterocolitica* outer membrane protein (*yop*) regulation and *yadA* gene involved in autoagglutination, serum resistance, and adhesion (Skurnik

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and Wolf-Watz, 1989). Therefore, rapid and accurate detection of pathogenic *Y. enterocolitica* should rely on virulent factors not only from the chromosome, but also from the plasmid.

Yersinia enterocolitica is able to survive and proliferate in frozen or refrigerated conditions and is widely distributed in milk samples (Schiemann, 1987; Yucel and Ulusoy, 2006; Hanifian and Khani, 2012). Frozen or refrigerated dairy products are popular with consumers. In China, 296 strains of *Y. enterocolitica* were collected from humans with diarrhea, pigs, rodents, sheep, and dogs between 1984 and 2011 for analysis of variation and changes in serotypes, biotypes, and genetic typing (Guo et al., 2012). In another paper, Huang et al. (2010) reported possible use of *ail* and *foxA* polymorphisms for detection of *Y. enterocolitica* strains isolated from humans with diarrhea, animals, and environments. However, information about prevalence of pathogenic *Y. enterocolitica* in frozen or refrigerated dairy products is not available.

The aim of this study was to develop a duplex PCR targeting the *virF* and *ail* genes for determination of prevalence of pathogenic *Y. enterocolitica* in refrigerated or frozen dairy products. In addition, we developed a dot hybridization assay based on *virF* or *ail* genes to further determine the specificity of the duplex PCR. Thereafter, the detection limits of the duplex PCR and dot hybridization were determined. Finally, virulent and avirulent *Y. enterocolitica* isolates were tested in commercial frozen or refrigerated dairy samples ($n = 88$) by the duplex PCR and the China National Standard (2008) method.

MATERIALS AND METHODS

Strains Used in this Study

All strains used in this study were from Guangdong Provincial Key Laboratory of Microbiology Culture Collection and Application (Guangzhou, China). Strains included 72 *Y. enterocolitica*, including 2 pathogenic strains (CMCC 52204 and CY39), 22 *Escherichia coli*, 16 *Salmonella*, 1 *Enterobacter cloacae*, 16 *Listeria monocytogenes*, 9 *Staphylococcus aureus*, 10 *Aeromonas hydrophila*, and 6 *Aeromonas veronii*. *Yersinia enterocolitica* CY39 and the type strain CMCC 52204 were used as positive controls for detection of *ail*⁺/*virF*⁺ and *ail*⁺, respectively. The non-*Yersinia enterocolitica* strains were used as negative controls.

Extraction of Genomic and Plasmid DNA of *Y. enterocolitica* and Other Strains

All species and strains listed above were inoculated into brain heart infusion broth (BHI, Huankai, Guang-

zhou, China) at 28°C for 36 h (for *Y. enterocolitica*) or 37°C for 24 h (for other strains), respectively. One milliliter of enrichment culture (about 6.5×10^9 cfu/mL for *Y. enterocolitica*, and 3.5 to 7.8×10^9 cfu/mL for other species) was used for extraction of genomic and plasmid DNA using the nucleic acid purification kit (DSBIO, Guangzhou, China) and high plasmid extraction kit (DSBIO), respectively, according to the manufacturer's instructions. The DNA templates were dissolved in Tris-EDTA buffer (30 μ L) and stored at -30°C until further use.

Detection of Pathogenic *Y. enterocolitica* by Duplex PCR

The primers were described previously by Thoerner et al (2003). For the duplex PCR, 2 pairs of primers were used: (forward): 5'-taatgtgtacgtgcgag-3', (reverse): 5'-gacgtcttacttgcaactg-3' for *ail* amplification; and (forward): 5'-ggcagaacagcagtcagacata-3', (reverse): 5'-ggtgagcatagagaatacgtcg-3' for *virF* amplification. The PCR mixture (25 μ L) consisted of 1.0 μ L of primers (10 μ M) for each, 200 μ M deoxynucleotide triphosphates, 2 U of *Taq* DNA polymerase, 2.5 μ L of 10 $\times$ buffer, 3 μ L of DNA template, and double-distilled water. The duplex PCR was performed as follows: denatured for 2.0 min at 95°C, followed by 35 cycles of 1.0 min at 94°C, 45 s at 58°C, and 1.0 min at 72°C; and final extension at 72°C for 7.0 min. The amplicons were detected by 1.5% agarose gel electrophoresis with Goldview staining (0.008%, vol/vol, Beijing Solarbio Science & Technology Co. Ltd., Beijing, China), and were photographed under a UV transilluminator.

Labeling and Detection of Pathogenic *Y. enterocolitica* by Dot Hybridization

The *virF* (561 bp) and *ail* (351 bp) sequences from the positive strains were labeled using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Basel, Switzerland) according to the manufacturer's instructions. The detection of dot hybridization was performed using Detection Starter Kit I (Roche) according to the manufacturer's instructions. In brief, 1.0 μ L of tested DNA template was spotted onto the nylon membrane, the membrane was placed onto 3-MM paper (Whatman PLC, Little Chalfont, UK) soaked with 2 $\times$ saline sodium citrate, and the template DNA on wet membrane was fixed by crosslinking with UV light. After crosslinking, the membrane was rinsed briefly in double-distilled water and was allowed to air dry. Thereafter, the membrane was transferred into a plastic container with DIG Easy Hyb (10 mL/100-cm² filter; Roche) for prehybridization with gentle agitation

for 30 min. Prehybridization solution was poured off and mixture of denatured labeled probe and preheated DIG Easy Hyb (3.5 mL/100-cm² filter) was transferred onto the membrane for incubation for 4 h. After hybridization and stringency washes, the membrane was briefly washed (5 min) in washing buffer, and then incubated in 10 mL of blocking solution for 30 min and 10 mL of antibody solution for 30 min, washed with 10 mL of washing buffer twice for 15 min each time, and then equilibrated in 10 mL of detection buffer for 2 to 5 min. Finally, the membrane was incubated in 2 mL of freshly prepared color nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt (NBT/BCIP) substrate solution in a hybrid tube in the dark. When desired spot or band intensities were achieved, the membrane was washed for 5 min with 50 mL of Tris-EDTA buffer to stop the reaction, and then the membrane was documented by photography.

Detection Limit for Artificially Contaminated Dairy Samples

Refrigerated yogurt (1.0 g) negative for *Y. enterocolitica* by the China National Standard (2008) method was mixed with 8 mL of sterile distilled water in a tube. Serial 10-fold dilutions from 6.5×10^9 to 6.5×10^0 cfu/mL were made, and 1.0 mL of each dilution was added to 9.0 mL of reconstituted yogurt. One milliliter of artificially contaminated yogurt was subject to genomic and plasmid DNA extraction using the same kits as used extraction of DNA from bacterial species. Genomic and plasmid DNA were stored at -30°C pending analysis. The PCR amplification and dot hybridization protocol were performed as described above.

Detection Limit of PCR Combined with Nonselective Enrichment

Brain heart infusion broth (5 mL) was inoculated with 4.5 to 5.7 cfu of *Y. enterocolitica* CY39 and incubated at 28°C with shaking (180 rpm) for 2, 4, 6, 8, 10, 12, 14, 16, and 18 h. The extraction of plasmid and genomic DNA from the different time-enrichment cultures (1.0 mL) and PCR amplification were performed as described above.

Detection of *Y. enterocolitica* in Commercial Frozen or Refrigerated Dairy

Eighty-eight frozen or refrigerated dairy samples including ice cream ($n = 20$), refrigerated yogurt ($n = 14$), milk popsicles ($n = 18$), refrigerated raw milk ($n = 14$), and frozen cheese ($n = 22$) were collected from supermarkets or retail shops. The samples (25 g) were

incubated into 225 mL of modified phosphate saline buffer and incubated at 28°C for 48 h. Then, 1 mL of culture was subjected to extraction of template DNA (genomic DNA and plasmid DNA). *Yersinia enterocolitica* and pathogenic *Y. enterocolitica* were isolated and detected by the China National Standard (2008) method and the duplex PCR method, respectively.

Phenotyping of Pathogenic *Y. enterocolitica* Strains

Yersinia enterocolitica phenotypes were grouped by biotyping (lipase, esculin, salicin, indole, xylose, and trehalose tests) and serotyped, using commercial serum, as O:3, O:5, O:8, O:9, and O:1, 2 (Denka Seiken, Tokyo, Japan) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Yersinia enterocolitica is a common foodborne pathogen causing human cases of yersiniosis through consumption of contaminated food (Tan et al., 2014). Consequently, detection of pathogenic *Y. enterocolitica* in frozen dairy samples is important in assuring microbial safety.

In this study, a duplex PCR targeting plasmid-borne *virF* and chromosome-borne *ail* genes was developed to simultaneously detect virulent *Y. enterocolitica* strains in frozen or refrigerated dairy samples. The expected fragments (561 bp for *virF*, 351 bp for *ail*) were successfully amplified from 12 out of 72 *Y. enterocolitica* isolates, but not in any non-*Y. enterocolitica* strains by our new duplex PCR. In Figure 1, among 12 pathogenic *Y. enterocolitica*, 6 pathogenic *Y. enterocolitica* strains were positive for both *ail* and *virF* genes, and 6 strains were positive for the *ail* gene only. As seen in Figure 2, detection by dot hybridization was consistent with results detected by the duplex PCR, which further confirmed the specificity of the duplex PCR method for detection of virulent *Y. enterocolitica*. Using the dot hy-

Figure 1. Detection of pathogenic *Yersinia enterocolitica* strains using duplex PCR. M = marker; lanes 1 to 6 = pathogenic *Y. enterocolitica* strains containing both *ail* and *virF* genes; lanes 1' to 6' = pathogenic *Y. enterocolitica* strains containing *ail* gene only; P = positive control, N = negative control.

Figure 2. Detection of pathogenic *Yersinia enterocolitica* strains by dot hybridization assay on *ail* and *virF* genes. A-*ail* = 6 pathogenic *Y. enterocolitica* strains containing *ail* gene only; B-*ail* and B-*virF* = 6 pathogenic *Y. enterocolitica* strains containing both *ail* and *virF* virulence genes; P = positive control, N = negative control. Dots 1 to 6 correspond to the 6 pathogenic *Y. enterocolitica* strains in each set.

bridization assay, we achieved a detection limit of 6.5×10^3 cfu/mL (Figure 3A). A detection limit of 1,000 cfu by microarray hybridization targeting virulence genes in pasteurized whole milk was described by Myers et al. (2006). As seen in Figure 3B, our duplex PCR could directly detect 6.5×10^2 cfu/mL of *Y. enterocolitica* in artificially contaminated dairy samples. A detection level of 4.0×10^2 cfu of *Y. enterocolitica* per mL of enrichment medium was obtained by a duplex PCR targeting *yadA* and 16S rRNA genes in combination with the density gradient centrifugation method (Lantz et al., 1998). Nilsson et al. (1998) reported that as few as 10^2 cfu could be successfully detected in ground pork in the presence of 10^5 to 10^6 cfu of other species by a single PCR targeting *ail* or *virF*. However, in that study, only 16 of 25 pathogenic *Y. enterocolitica* strains simultaneously yielded positive results for both genes. To determine the influence of enrichment on the detection limit (Figure 4), the expected amplicons were amplified by the duplex PCR from the artificially dairy sample inoculated with 4.5 to 5.7 cfu of *Y. enterocolitica* /5 mL of brain heart infusion broth after 6 h of nonselective enrichment at 28°C. In an earlier study using selective enrichment at 25°C, a positive PCR result was obtained from selective enrichment medium with a background flora inoculated with approximately 1.0×10^1 cfu/mL *Y. enterocolitica* after 6 to 8 h at 25°C (Lantz et al., 1998). The current study also indicated that the duplex PCR was specific and sensitive for detection of pathogenic *Y. enterocolitica* when combined with an enrichment step.

Surveillance of *Y. enterocolitica* is important for risk analysis and assessment of frozen or refrigerated dairy products. In Pennsylvania, *Y. enterocolitica* was detected in 248 (1.2%) milk samples (Jayarao et al., 2006). In northwestern Iran, 7.6% of bulk raw milks ($n = 354$) and 10.5% of retail cheeses ($n = 200$) was found to be positive for *ail* by PCR (Hanifian and Khani, 2012). In Turkey, a survey indicated that *Y. enterocolitica* was

Figure 3. Detection limit of *Yersinia enterocolitica* strains in artificially contaminated dairy product by dot hybridization (A) and duplex PCR (B) for *ail* and *virF* genes. (A) dots 1 to 6 = 6.5×10^6 , 6.5×10^5 , 6.5×10^4 , 6.5×10^3 , 6.5×10^2 , and 6.5×10^1 cfu/mL, respectively; (B) M = marker; lanes 1 to 6 = 6.5×10^7 , 6.5×10^6 , 6.5×10^5 , 6.5×10^4 , 6.5×10^3 , and 6.5×10^2 cfu/mL, respectively.

isolated from 47.3% of raw milk samples and 35.7% of cheese samples (Yucel and Ulusoy, 2006). In the current study (Table 1), out of 88 dairy samples, pathogenic *Y. enterocolitica* was detected in 5 (5.7%) samples, of which milk popsicles had the highest prevalence (2/18; 11.1%), followed by refrigerated raw milk (1/14; 7.1%), ice cream (1/20; 5%), frozen cheese (1/22; 4.5%), and refrigerated yogurt (0/14; 0.0%), compared with 16.7, 15, 14.3, 14.3, and 13.6% of milk popsicles, ice cream, refrigerated yogurt, refrigerated raw milk, and frozen cheese samples shown to be positive for *Y. enterocolitica* by the China National Standard (2008) method. In Table 2, phenotypic tests indicate that 17 strains were grouped into biotype 1B (7 isolates), biotype 2 (8 isolates), and biotype 3 (2 isolates). Miller et al. (1989) reported that *Y. enterocolitica* from biotypes 1B, 2, 3, 4, and 5 were pathogenic in humans, which was consistent with our results. The following O serotypes were detected among these pathogenic *Y. enterocolitica* strains: O:8 (7 isolates), O:5 (4 isolates), O:3 (3 isolates), and O:9 (2 isolates), but 1 isolate was negative for the O serotype (O:3, O:5, O:8, O:9, and O:1, 2). Serotype groups associated with human infections (O:3, O:5, O:8, and O:9) were previously described by Botone (1999), but a clear correlation between biotype and O serotype was not observed in this study.

The survey in commercial dairy samples indicated that contamination of *Y. enterocolitica* is relatively severe and should be emphasized. In addition, only 1 of 5 pathogenic *Y. enterocolitica* strains bore both the *ail* and *virF* genes. Previous studies have reported that all *Y. enterocolitica* strains harboring the *ail* gene always carry the *yst* gene (Miller et al., 1989; Harnett et al., 1996; Myers et al., 2006). In present study, we found that the plasmid-borne *virF* did not occur alone in pathogenic *Y. enterocolitica* and was always accompanied by the *ail* gene. The developed duplex PCR was able to differentiate pathogenic *Y. enterocolitica* from apathogenic strains regardless of biotype or serotype. The serotypes O:3, O:5, O:8, and O:9 and biotypes 1B, 2, 3, 4, and 5 were considered pathogenic (Wan-

Figure 4. Detection of duplex PCR for *ail* and *virF* genes combined with nonselective enrichment. M = marker; N = negative control; lanes 1 to 8 = enrichment for 18, 16, 14, 12, 10, 8, 6, and 4 h at 28°C, respectively.

net et al., 2001; Hanifian and Khani, 2012), but the relationship between these phenotypes and virulence is not completely consistent (Thoerner et al., 2003). The method described here could also distinguish pathogenic *Y. enterocolitica* strains with or without the virulence plasmid harboring the *virF* gene. Furthermore, instability of the virulence plasmid has been reported previously (Thoerner et al., 2003; Myers et al., 2006) and the plasmid can be easily lost under conditions of alkaline pH (e.g., pH 9.0; data not shown). Consequently, plasmid-borne virulence genes are not appropriate as the sole marker for specifically detecting pathogenic *Y. enterocolitica* strains in food samples.

CONCLUSIONS

In this study, we developed a duplex PCR targeting *ail* and *virF* virulence genes that could specifically and sensitively detect pathogenic *Y. enterocolitica* in frozen or refrigerated dairy products. A dot hybridization assay further confirmed the specificity of the duplex PCR. When combined with an enrichment step, the duplex PCR had high sensitivity. Detection of pathogenic *Y. enterocolitica* in commercial frozen or refrigerated dairy samples indicated that the duplex PCR was reli-

Table 1. Detection of *Yersinia enterocolitica* and pathogenic *Y. enterocolitica* in commercial refrigerated or frozen dairy samples by duplex PCR and China National Standard (2008; GB/T4789.8-2008)

Dairy sample	Detection by duplex PCR ¹			GB/T4789.8-2008 method
	<i>ail</i>	<i>virF</i>	<i>ail</i> and <i>virF</i>	
Refrigerated raw milk (14)	1	0	0	2
Ice cream (20)	1	1	1	3
Milk popsicles (18)	2	0	0	3
Refrigerated yogurt (14)	0	0	0	2
Frozen cheese (22)	1	0	0	3
Total (88)	5	1	1	13

¹*ail* = chromosome-borne attachment invasion locus gene; *virF* = plasmid-borne virulence factor F gene.

Table 2. Virulence factors and phenotyping of pathogenic *Yersinia enterocolitica* isolates

<i>Y. enterocolitica</i> strain ¹	Virulence factor ²	Biotype	Serotype	Origin (food and region)
Ye1,Ye3	<i>ail</i>	1B	O:3	Meat (Xianan), chicken (Hefei)
Ye4,Ye5, Ye6	<i>ail</i>	2	O:5	Frozen dumpling (Haerbing), meat (Kunming), meat gruel (Shanghai)
Ye2	<i>ail</i>	3	ND ³	Frozen chicken (Jinan)
Ye7, Ye10, Ye12	<i>ail/virF</i>	2	O:8	Frozen meat (Xianan), pork (Shantou), frozen chicken (Lanzhou)
Ye8	<i>ail/virF</i>	1B	O:9	Meat (Wuhan)
Ye9, Ye11	<i>ail/virF</i>	1B	O:8	Frozen meat (Lanzhou), meat gruel (Shanghai)
N [▲] -Ye1,N [▲] -Ye2	<i>ail</i>	1B	O:8	Milk popsicles (Hefei), refrigerated raw milk (Hefei)
N [▲] -Ye3	<i>ail</i>	3	O:9	Milk popsicles (Hefei)
N [▲] -Ye4	<i>ail/virF</i>	2	O:3	Ice cream (Hefei)
N [▲] -Ye5	<i>ail</i>	2	O:5	Frozen cheese (Hefei)

¹N[▲] = newly isolated from frozen and refrigerated dairy samples.
²*ail* = chromosome-borne attachment invasion locus gene; *virF* = plasmid-borne virulence factor F gene.
³ND = nondetectable for O:3, O:5, O:8, O:9, and O:1, 2.

able for detection of pathogenic *Y. enterocolitica* and for routine inspection and monitoring in refrigerated or frozen dairy samples.

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