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Development of a novel multiplexed qPCR and Pyrosequencing method for the detection of human pathogenic yersiniae

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ABSTRACT

The purpose of this study was to develop a novel and robust molecular assay for the detection of human pathogenic yersiniae (i.e. *Yersinia enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*) in complex food samples. The assay combines multiplexed real-time PCR (qPCR) and Pyrosequencing for detecting and differentiating human pathogenic yersiniae with high confidence through sequence based confirmation. The assay demonstrated 100% specificity and inclusivity when tested against a panel of 14 *Y. enterocolitica*, 22 *Y. pestis*, 24 *Y. pseudotuberculosis* and a diverse selection of 17 other non-*Yersinia* bacteria. Pyrosequencing reads ranged from 28 to 40 bp in length and had 94–100% sequence identity to the correct species in the GenBank nr database. Microbial enrichments of 48 ready-to-eat foods collected in the Greater Toronto Area from March 2014 to May 2014, including 46 fresh sprout and 2 salad products, were then tested using the assay. All samples were negative for *Y. pestis* and *Y. pseudotuberculosis*. Both salads (n = 2) and 35% of sprout products (n = 46) including 7.1% of alfalfa sprouts (n = 14), 81% of bean sprouts (n = 16), 12% of mixed sprouts (n = 8) tested positive for *Y. enterocolitica* which was not detected in broccoli sprouts (n = 5), onion sprouts (n = 1), and pea sprouts (n = 2). Cycle thresholds (Ct) of positive samples for *Y. enterocolitica* were between 23.0 and 37.9 suggesting post enrichment concentrations of approximately 1×10^2 to 1×10^6 *Y. enterocolitica* per 1 mL of enriched broth. An internal amplification control which was coamplified with targets revealed PCR inhibition in five samples which was resolved following a one in ten dilution. Pyrosequencing of qPCR amplicons suggests monoclonality and revealed a single nucleotide polymorphism that is present in *Y. enterocolitica* biotype 1A suggesting low pathogenicity of the detected strains. This study is the first to combine Pyrosequencing and qPCR for the detection of human pathogenic yersiniae and is applicable to a broad range of complex samples including ready-to-eat food samples.

1. Introduction

Yersinia is a diverse genus of Gram negative rods in the Enterobacteriaceae family containing 18 species, 3 of which, including *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* are pathogenic in humans. *Y. enterocolitica* and *Y. pseudotuberculosis* are enteropathogens that cause yersiniosis, a self-limiting form of bacterial gastroenteritis (McNally et al., 2016). *Y. pestis* is the etiologic agent of plague and is a recently emerged clone of *Y. pseudotuberculosis* regarded for its potential as a biothreat agent due to its high mortality rate among infected individuals and low infectious dose (Achtman et al., 1999). *Y. enterocolitica* and *Y. pseudotuberculosis* are recognized as important foodborne pathogens and are of particular concern due to their ability to replicate at food refrigeration temperatures (Giannitti et al., 2014; Söderqvist et al., 2017). The pathogens have been implicated in numerous outbreaks and are acquired most often by ingesting contaminated pork but have also been associated with raw milk and occasionally vegetable products (Longenberger et al., 2014; MacDonald et al., 2012). Yersiniosis is the third most frequently reported zoonosis in Europe, with an incidence rate of 1.92 cases per 100,000 people, in which most cases are caused by *Y. enterocolitica* (European Food Safety Authority European Centre for Disease Prevention and Control, 2014). While yersiniosis is not on the list of nationally notifiable diseases in Canada, provincial data from British Colombia and Ontario in the 2011–2012 FoodNet Canada report suggests an incidence rate of 2.91 cases per 100,000 person-years (Public Health Agency of Canada, 2015).

In 2009, consumption of fresh fruits and vegetables (excluding potatoes) in Canada reached record levels of 39.3 and 40.7 kg per person, respectively (Statistics Canada, 2010). While important for maintaining

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a healthy lifestyle, fresh fruits and vegetables are recognized as vectors for food borne illnesses. Ready-to-eat (RTE) foods are a broad category of food stuffs including many fresh fruits and vegetables and are defined as any item of food consumed in its raw state (Cerna-Cortes et al., 2015). They are produced and distributed on a global scale from centralized locations and thus recalls and outbreaks associated with RTE foods can be at the national and even international levels as seen with 2011 *Escherichia coli* O104:H4 outbreak in Europe (Karch et al., 2012) and the North American outbreak of *Salmonella enterica* in cantaloupe (Huang et al., 2015). Ready-to-eat foods can become contaminated at multiple stages of their production including in the field, during primary processing (e.g. washing), and final preparation. Ready-to-eat sprouts and salads are especially vulnerable as they are difficult to disinfect without impacting germination and/or overall food quality. Although human pathogenic yersiniae are seldom targeted for detection in RTE foods, RTE salad has been associated with an outbreak of *Y. enterocolitica* in Norway (MacDonald et al., 2012) and the ability of these organisms to grow under the modified atmosphere inside packaging and at food refrigeration temperatures are of special concern for this food type (Fukushima et al., 2011).

The detection of human pathogenic yersiniae has previously been accomplished by both culture dependent (Premaratne et al., 2012) and more recently, culture independent techniques (Amoako et al., 2012a; Lambertz et al., 2008; Mäde et al., 2008; Siddique et al., 2009; Wang et al., 2014). Culture independent techniques such as PCR, real time quantitative PCR (qPCR), and Pyrosequencing are typically faster and more cost effective than traditional methods and can be used as screening and confirmatory tools. These methods are inherently sensitive and with careful design can be extremely specific due to the recent expansion of online sequence databases such as NCBI resulting from advances in whole genome sequencing technologies and their widespread adoption. The majority of culture independent detection studies, with the exception of metagenomic studies, have focused on detection of a single organism and to our knowledge, no assays have been developed to screen for and differentiate human pathogenic yersiniae simultaneously. Utilizing qPCR and Pyrosequencing in tandem allows for a quantitative assessment of the level of contamination as well as rapid sequence confirmation which can be designed in a way to yield genotype information for isolate via single nucleotide polymorphisms (SNPs) and insertions or deletions (indels).

The objective of the present study was to design a novel qPCR and Pyrosequencing assay capable of detecting and differentiating human pathogenic yersiniae. The developed assay was then applied to routine surveillance samples at the Canadian Food Inspection Agency including ready-to-eat sprouts and salads.

2. Materials and methods

2.1. Extraction and quantification of genomic DNA

Genomic DNA was extracted from bacteria using the DNeasy Blood & Tissue kit (Qiagen Inc., Mississauga, ON, Canada) as detailed previously (Amoako et al., 2010). Briefly, *Yersinia* spp. were grown overnight in 5 mL of brain heart infusion (BHI) broth (BD Difco, Burlington, ON, Canada) at 28 °C. Non-*Yersinia* spp. were grown in 5 mL BHI overnight at 37 °C. Following overnight growth, 2 mL of broth was centrifuged at 10,000 × *g* for 1 min to pellet cells, the supernatant discarded, and DNA extracted from the cell pellet. Extracted DNA was quantified using the Nanodrop ND-8000 spectrophotometer (Nanodrop Inc., DE, USA) and diluted to 5 ng/μL for use as PCR template.

2.2. Design of oligonucleotide primers and probes specific for Yersinia species

Oligonucleotide primers targeting *Y. pestis* were designed previously and published elsewhere (Amoako et al., 2010) while the probe was designed as part of this study. Novel primers and probes were also designed for *Y. pseudotuberculosis* and *Y. enterocolitica* using Geneious R7 (Biomatters Inc., CA, USA) and MPprimer (Shen et al., 2010). The *Y. pseudotuberculosis* target region was chosen based on a region recently identified to be conserved in *Y. pseudotuberculosis* yet absent from other yersiniae (Pouillot et al., 2008). For the design of a novel *Y. enterocolitica* target, whole genome sequences for *Yersinia enterocolitica* LC20, *Y. pseudotuberculosis* ATCC 6904 and *Y. pestis* CO92 were downloaded from the NCBI database and aligned using the MAUVE plugin for Geneious R7 (version 7.1.4; Biomatters Inc. [http://www.geneious.com]) (Darling et al., 2010; Kearse et al., 2012). Characteristic regions were chosen as candidates for primer design for identification and differentiation of the three bacterial species. Candidate regions were blasted against the NCBI nr nucleotide database and sequences demonstrating specificity and inclusivity were selected for design of oligonucleotide primers and probes (Table 1). Oligonucleotide primers, one of which was biotinylated for each target, and Taqman probes were synthesized by Integrated DNA Technologies (IDT; Iowa, USA) with the exception of the LC610 probe which was synthesized by TIB MOLBIOL (Berlin, Germany). Taqman probes were synthesized with 5′ modifications of Cy5, 6-FAM, and LC610 and the corresponding 3′ quenchers Iowa Black® RQ, 3′ Black Hole Quencher®-1, and BlackBerry® Quencher for *Y. enterocolitica*, *Y. pestis*, and *Y. pseudotuberculosis*, respectively.

Table 1
Primers and probes for each target organism in the *Yersinia* speciation multiplex.

Target organism	Primer	Sequence (5′ - 3′)	Reference
Yersinia enterocolitica	YE_outL_1F[a]	CATCATCCAGCTTCACTGGAAAGCA	This study
	YE_outL_1R-b[b]	/5Biosg/GTTGGTTGGGGTGGTTAACAAATGTAG	This study
	YE_outL_mP	/5Cy5/CGGAATCATGACGGTGGGAGAGA/3IAbRQSp/	This study
Yersinia pestis	YP_c4_1F	TGTGTGGTCTAGCAAAGCTTATGACG	Amoako et al. (2010)
	YP_c4_1R-b	/5Biosg/CGAACGAAAGCGGAAAAGTGAGGAT	Amoako et al. (2010)
	YP_c4_1S[c]	GCAAAGCTTATGACGTCCTTTCTTG	Amoako et al. (2010)
	YP_c4_P	/5Cy3/ATGTGTGGTCTAGCAAAGCTTATGACGTCC/3BHQ_2/	This study
Yersinia pseudotuberculosis	YPTB_2199_1F	CTTTTTACCTTGGCGCTTTTGCTGG	This study
	YPTB_2199_1R-b	/5Biosg/ACAAGAGAGGCCTGAAAAGCATTGG	This study
	YPTB_2199_1S[c]	GCGCTTTTGCTGGAGTATTTGG	Amoako et al. (2010)
	YPTB_2199_mP	LC610-TGTGCTATTTTCTGCTCCAACAAAAGG-BBQ	This study

[a] The forward primer (F) was also used as the sequencing primer.
[b] This primer was 5′ biotinylated.
[c] This primer was used as the sequencing primer during Pyrosequencing.