Development of a fluorogenic 5' nuclease PCR Assay for the detection of pathogenic *Yersinia enterocolitica*.

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Summary and Implications

In this report, we describe the development and evaluation of a 5' nuclease PCR assay for the detection of pathogenic *Yersinia enterocolitica* (YE). The assay targets the chromosomally encoded invasion gene *ail*. Three different primer/probe sets (TM1, TM2, and TM3) amplifying different, yet overlapping, regions of *ail* were examined for their specificity and sensitivity. The TM1 set displayed the highest specificity, accurately detecting each of the 26 YE strains and none of the 21 non-*enterocolitica* strains. This set was sensitive to approximately 0.5 pg of purified *Y. enterocolitica* DNA. The TM2 set was the most sensitive, allowing detection in the range of 0.25 pg of purified DNA. However, it was not specific and failed to recognize 10 of the YE strains used in this study. Sensitivities comparable to TM1 were achieved with the TM3 set; cross-reaction with non-*enterocolitica* strains was not observed. However, this set failed to positively identify all of the YE strains tested.

Introduction

YE is a food-borne pathogen and estimated to cause 3,000 to 20,000 cases of human disease annually in the United States (8). Clinical manifestations typically include abdominal pain, fever, diarrhea, and nausea (2,7). The disease can range from a self-limiting gastroenteritis to a potentially fatal septicemia (2). YE strains are found in both aquatic and animal reservoirs (2,9). However, healthy swine are the only animals known to harbor human pathogenic YE (1). To accurately monitor the prevalence of YE in animals and animal products rapid, specific, and sensitive methods of identification are required.

Polymerase chain reaction (PCR) is a powerful tool for the detection and identification of organisms in animals and food products, including pathogenic YE. However, conventional PCR assays typically require lengthy enrichment protocols, followed by PCR amplification and gel-based detection. Newer PCR protocols no longer require gel-based detection, but instead rely on cleavage of a fluorogenic probe for automated and specific detection amplicons. The 5' nuclease PCR assay is more specific and sensitive than conventional PCR in detecting pathogens (3,5,6). In addition, the 5' nuclease assay is performed in a 96-well format such that sampling can be automated reducing sample handling time and minimizing cross contamination. Furthermore, the assay can be quantitative, allowing for the enumeration of target present in a given unknown sample.

The 5' nuclease assay utilizes the inherent 5' → 3' nuclease activity of *Taq* DNA polymerase to cleave an internal fluorogenic probe (consisting of an oligonucleotide with both a reporter and a quencher dye). During PCR, the fluorogenic probe anneals to the target DNA between the two primers and is cleaved during amplification by the 5' nuclease activity of *Taq* DNA polymerase. Cleavage releases the fluorescent reporter from the probe and the attached quencher, thus increasing the fluorescent emission of the reaction. Consequently, an increase in fluorescent emission indicates amplification of target DNA.

In this report we describe the development and evaluation of the sensitivity and specificity of a 5' nuclease assay for amplifying the *ail* locus and presumptively detecting YE. Evaluation of the nuclease assay included optimization of reaction conditions for each of 3 primer/probe sets.

Materials and Methods

Bacterial strains and culture conditions. The strains listed in Table 1 were obtained from the National Animal Disease Center (NADC), the American Type Culture Collection (ATCC), and the Centers for Disease Control (CDC). Strain NADC 5571 is a YE strain isolated from a yersiniosis outbreak from human consumption of contaminated chitterlings. *Yersinia* strains were grown in either tryptic soy broth (Difco) or ITC broth (ticarcillin-irgasan-potassium chlorate) (10) overnight at 30˚C with shaking.

DNA isolation. Genomic DNA was isolated from bacterial cells using a modified guanidine/silica particle extraction protocol (4). Briefly, 1 ml of bacterial culture was pelleted (1 min at 14,000 rpm). The bacterial pellet was resuspended in 0.5 ml of diatom DNA binding solution (1% diatomaceous earth, 6 M guanidine HCl), frozen at -70°C to lyse the cells, thawed, and the diatoms pelleted (1 min, 14,000 rpm). The diatom pellet was washed with 95% ethanol and the DNA eluted by adding 50 µl dH_20 and heating at 65°C for 10 min. RNase A was added to each DNA sample at a

concentration of 1 mg/ml and incubated at 37˚C for 15 min. The DNA concentration of each sample was determined spectrophotometrically at 260 nm.

YE strains NADC 5231 and 5560 were used as standards in determining the sensitivity of the assay. DNA samples were brought to a concentration of 0.1 µg/µl then serially diluted 10-fold to 1 fg/µl. The final range of DNA concentrations was from 500 ng to 50 fg per 50 µl reaction. The 47 *Yersinia* DNA samples used in the specificity testing were normalized to a concentration of 10 ng/µl and were present in reactions at a final concentration of 1 ng/µl. All DNA samples were stored at -20˚C.

Primers and probes. Three independent sets of primers and probes specific for the YE *ail* gene were designed using Primer Express™ software (PE ABI Prism). Each of the primer/probe sets amplified a different, yet overlapping, region of the *ail* gene. Each probe was labeled at the 5' end with the fluorescent reporter dye FAM (6-carboxy-fluorscein) and at the 3' end with the quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine).

5' nuclease PCR conditions. PCR reaction conditions were as follows: 3.5 mM $MgCl_2$, 0.2 mM dNTP's, GeneAmp® PCR Gold Buffer (PE Biosystems), 1.25 U of AmpliTaq Gold™ DNA polymerase, and 5 µl of DNA template in a total volume of 50 µl. Each of the primers was added at a concentration of 200 nM. Probe concentrations were as follows: TM1, 25 nM; TM2, 50 nM; and TM3, 100 nM. Thermal cycling conditions 95˚C for 10 min, 35 cycles of 95˚C for 15 seconds and 58˚C for 1 min followed by an indefinite hold at 25˚C.

Data analysis. PCR reactions were performed in a 96-well format in the PE ABI PRISM™ 7700 Sequence Detection System (PE Applied Biosystems) and Sequence Detector™ software version 1.6.3 (PE Applied Biosystems) was used in the data analysis.

Results and Discussion

Assay optimization and sensitivity testing. The 5' nuclease PCR assay was optimized for each primers/probe set by testing a range of $MgCl_2$, primer, and probe concentrations as well as the number of amplification cycles. An optimal primer concentration of 200 nM was determined for each of the three primer sets. However, the ideal probe concentration differed, possibly indicating differences in probe annealing affinities or cleavage rates. The optimum cycle number (35) and $MgCl_2$ concentration (3.5 mM) were identical for each primer/probe set.

To assess the minimum amount of YE DNA detectable by each of the sets, serial dilutions of known concentrations of YE DNA were tested. The TM2 set could detect 0.50 pg of purified YE DNA, whereas the TM2 and TM3 sets required approximately two-fold more DNA to generate a positive signal.

Specificity testing. Twenty-six YE strains and nine *Yersinia* sp., for a total of 47 *Yersinia* strains (Table 1) were examined. Each of the primer/probe sets were tested for their ability to positively identify YE. The TM1 set was the most specific, amplifying all the YE and none of the non-*enterocolitica* strains. TM2 and TM3 , however, were not as specific and failed to recognize all of the *Y. enterocolitica* strains (Table 1). Despite their inability to detect all YE strains, neither TM2 or TM3 displayed any cross-reactivity with the non-*enterocolitica* strains under optimal PCR cycling conditions. Current investigations focus on determining the minimum CFU/ml detection level with the TM1 set. Preliminary data indicates that less than 10 CFU/ml can be accurately detected.

The 5' nuclease PCR assay is a promising tool for the rapid, sensitive, specific, and automated detection of YE. The TM1 set of primers/probe proved to be the most specific, detecting all YE and not cross-reacting with any non-*enterocolitica*. Although this set was not the most sensitive, it is still two to three orders of magnitude more sensitive than a YE multiplex PCR developed in this laboratory. Furthermore, the level of sensitivity achieved with the TM1 set would allow for shorter enrichments, thus providing a more rapid means of identification.

The 5' nuclease assay allows for quantification of PCR products. We have generated a standard curve for YE using highly purified DNA samples. According to these standards, very low amounts of YE DNA can be accurately and reproducibly detected. However, when this "clean" DNA is used as a standard to quantify "dirty" DNA prepared from field samples, accuracy is reduced. Therefore, we are currently developing a standard curve using dirty DNA that will more closely reflect the condition of YE DNA extracted from animal tissues and food samples.

We encountered some unexpected results while developing this assay that are worth noting. First, each of the primer/probe sets amplified different YE strains (having the same DNA concentration per reaction) with varying efficiencies. This could be for several reasons. The genomic DNA preps could contain low levels of DNA binding proteins, possibly obstructing annealing of the primers or probes, reducing the amount of amplification and thus fluorescent signal. Another possibility is that the region of *ail* targeted by the primer/probe sets vary slightly between different YE strains. Again, this could decrease primer or probe annealing and consequently the amplification signal. Second, when PCR reactions containing either TM1, TM2, or TM3 are allowed to amplify above cycle 35 some of the non-*enterocolitica* strains generate a positive signal. *Y. pseudotuberculosis* has been shown to contain a homologous *ail* locus, which could account for this result. However, currently there is insufficient sequence data on the other *Yersinia* species. Therefore, whether or not these species contain *ail* is unknown. However, the results obtained during development of this assay would indicate that *Yersinia* species other than *enterocolitica* and *pseudotuberculosis* may harbor the *ail* gene or a degenerate

version of this gene. We are currently investigating solutions to these assay inconsistencies.

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Table 1. Specificity of fluorogenic 5' nuclease assay for detecting *Yersinia enterocolitica.*

Strain	Number	Serotype	Origin[a]	Amplification TM1	TM2	TM3
Yersinia bercovieri	5230		ATCC	-	-	-
Yersinia enterocolitica	5231		ATCC	+	+	+
Yersinia enterocolitica	5232		ATCC	+	-	+
Yersinia enterocolitica	5233		ATCC	+	+	+
Yersinia enterocolitica	5234		ATCC	+	+	+
Yersinia enterocolitica	5235		ATCC	+	-	+
Yersinia kristensensii	5236		ATCC	-	-	-
Yersinia enterocolitica	5237		ATCC	+	+	-
Yersinia enterocolitica	5559	O:4,32	CDC	+	+	+
Yersinia enterocolitica	5560	O:8	CDC	+	+	+
Yersinia enterocolitica	5561	O:9	CDC	+	+	+
Yersinia enterocolitica	5562	O:18	CDC	+	-	+
Yersinia enterocolitica	5563	O:20	CDC	+	+	+
Yersinia enterocolitica	5564	O:21	CDC	+	+	+
Yersinia enterocolitica	5565	O:13	CDC	+	+	+
Yersinia enterocolitica	5566	O:5,27	CDC	+	+	+
Yersinia enterocolitica	5567	O:1,2,3	CDC	+	-	+
Yersinia enterocolitica	5568	O:2,3	CDC	+	-	+
Yersinia enterocolitica	5569	O:3	CDC	+	+	+
Yersinia enterocolitica	5570	O:3 H	CDC	+	+	+
Yersinia enterocolitica	5571		CDC	+	-	+
Yersinia enterocolitica	5610		ATCC	+	+	+
Yersinia enterocolitica	5611		ATCC	+	+	+
Yersinia aldovae	5612		ATCC	-	-	-
Yersinia aldovae	5613		ATCC	-	-	-
Yersinia aldovae	5614		ATCC	-	-	-
Yersinia bercovieri	5615		ATCC	-	-	-
Yersinia frederiksenii	5616		ATCC	-	-	-
Yersinia frederiksenii	5617		ATCC	-	-	-
Yersinia frederiksenii	5618		ATCC	-	-	-
Yersinia intermedia	5619		ATCC	-	-	-
Yersinia intermedia	5620		ATCC	-	-	-
Yersinia intermedia	5621		ATCC	-	-	-
Yersinia kristensensii	5622		ATCC	-	-	-
Yersinia kristensensii	5623		ATCC	-	-	-
Yersinia kristensensii	5624		ATCC	-	-	-
Yersinia mollaretii	5625		ATCC	-	-	-
Yersinia pseudotuberculosis	8119		ATCC	-	-	-
Yersinia pseudotuberculosis	8120		ATCC	-	-	-
Yersinia pseudotuberculosis	8121		ATCC	-	-	-
Yersinia ruckeri	8122		ATCC	-	-	-
Yersinia ruckeri	8123		ATCC	-	-	-
Yersinia enterocolitica	8177		Swine	+	+	+
Yersinia enterocolitica	8178		Swine	+	-	+
Yersinia enterocolitica	8179		Swine	+	-	+
Yersinia enterocolitica	8180		Swine	+	-	+
Yersinia enterocolitica	8181		Swine	+	-	+

[a] See Materials and Methods for abbreviation descriptions.