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Rapid 5′ Nuclease (TaqMan) Assay for Detection of Virulent Strains of *Yersinia enterocolitica*†

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We have developed a rapid procedure for the detection of virulent *Yersinia enterocolitica* in ground pork by combining a previously described PCR with fluorescent dye technologies. The detection method, known as the fluorogenic 5′ nuclease assay (TaqMan), produces results by measuring the fluorescence produced during PCR amplification, requiring no post-PCR processing. The specificity of the chromosomal *yst* gene-based assay was tested with 28 bacterial isolates that included 7 pathogenic and 7 nonpathogenic serotypes of *Y. enterocolitica*, other species of *Yersinia* (*Y. aldovae*, *Y. pseudotuberculosis*, *Y. mollaretti*, *Y. intermedia*, *Y. bercovieri*, *Y. ruckeri*, *Y. frederiksenii*, and *Y. kristensenii*), and other enteric bacteria (*Escherichia*, *Salmonella*, *Citrobacter*, and *Flavobacterium*). The assay was 100% specific in identifying the pathogenic strains of *Y. enterocolitica*. The sensitivity of the assay was found to be ≥10^2 CFU/ml in pure cultures and ≥10^3 CFU/g in spiked ground pork samples. Results of the assay with food enrichments prespiked with *Y. enterocolitica* serotypes O:3 and O:9 were comparable to standard culture results. Of the 100 field samples (ground pork) tested, 35 were positive for virulent *Y. enterocolitica* with both 5′ nuclease assay and conventional virulence tests. After overnight enrichment the entire assay, including DNA extraction, amplification, and detection, could be completed within 5 h.

Yersinia enterocolitica is a foodborne pathogen which causes gastrointestinal disorders with a wide range of clinical manifestations, from mild diarrhea to mesenteric lymphadenitis (5). The association of human illness with consumption of *Y. enterocolitica*-contaminated food, animal wastes, and unchlorinated water is well documented. This organism is known to contaminate refrigerated foods due to its psychotrophic nature and is frequently associated with ground pork and other ground meats. The species comprises a heterogeneous group of organisms with more than 50 serotypes and several biotypes (27). However, the pathogenic serotypes commonly associated with human yersiniosis are limited to European strains (O:1,3; O:3; O:9; and O:5) (26) and American strains (O:8; O:13a,13b; O:20; O:21; O:18; and O:4) (31). Virulence in *Y. enterocolitica* results from a complex interplay between a series of plasmid-borne and chromosomal genes (7, 20, 32). The latter include *yst*, the chromosomal gene encoding a low-molecular-weight, heat-stable enterotoxin, characteristic only of the virulent strains of *Y. enterocolitica* (10, 27). Pathogenic *Y. enterocolitica* strains are characterized by their ability to adhere to and invade epithelial cells (21, 26). This function is encoded by a genetic locus on the chromosome known as the *ail* gene (20). Once localized within the target cells, *Y. enterocolitica* is able to resist the primary immune response of the host. This resistance depends on the presence of a 70-kb virulence plasmid, pYV, which directs secretion of two major groups of proteins, called Yops and YadA, that are known to interfere with the functioning of phagocytic cells. Most human isolates of *Y. enterocolitica* produce a heat-stable enterotoxin with properties similar to those of the heat-stable enterotoxin of enterotoxigenic *Escherichia coli* (1).

Phenotypic differentiation between virulent and avirulent strains of *Y. enterocolitica* requires a series of biochemical and serological tests that are time consuming and that produce inconsistent results (23). Several investigators have developed PCR and DNA probe techniques for the detection of pathogenic *Yersinia* (9, 10, 14, 19, 22, and 28). The PCR technique has shown great promise as a highly sensitive and specific method but has several limitations. The amplified product must be detected in order to prove its presence, and a variety of methods, like gel electrophoresis and Southern blotting and dot blot hybridizations with labeled (chemical or radioactive) probes, have been used. However, these methods are time consuming and laborious, requiring skill and multistep processing that add to the cost and complexity of the test. The number of samples that can be analyzed at any one time is also limited. Furthermore, ethidium bromide, which is used to stain agarose gels, is a mutagen and is not appropriate for routine use in food-monitoring laboratories (24).

Recently, 5′ nuclease assays have been described that allowed the automated PCR amplification, detection, and analysis of *Salmonella* spp. (4, 13, and 18), *Listeria monocytogenes* (2, 3), *E. coli* O157:H7 (24), and Shiga-like toxin genes (30; M. S. Y. Ho, S. J. A. Flood, and C. Paszko-Kolva, Abstr. 97th Gen. Meet. Am. Soc. Microbiol. 1997, abstr. P-17, p. 439, 1997) in various foods. The 5′ assay exploits the 5′→3′ activity of *Thermus aquaticus* DNA polymerase (8, 17) to hydrolyze an internal TaqMan probe labeled with a fluorescent reporter dye and a quencher dye (16). The probe is designed to hybridize to an internal region of the targeted sequence. For the intact probe, the fluorescence from the reporter is suppressed by the quencher dye due to its spatial proximity to the reporter. As the PCR amplification proceeds, the annealed probe is hydrolyzed by the *Taq* DNA polymerase, separating the two dyes and increasing the reporter fluorescence signal that can be detected

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on a fluorometer (ABI Prism 7200 sequence detection system; PE Applied Biosystems). Because the increase in fluorogenic reporter signals is a direct consequence of a successful PCR, this procedure can be used in the detection of specific DNA sequences. The fluorometric data can be automatically read and interpreted using a 96-sample format and presented as positive or negative conclusions as to the presence or absence of the DNA within 15 min of completion of the PCR.

In this report, we describe the development and validation of a 5′ nuclease assay for the rapid (within 24 h) detection and analysis of virulent *Y. enterocolitica* in ground pork. The assay targets the heat-stable enterotoxin gene (*yst*), characteristic only of the virulent strains of *Y. enterocolitica*. The sequences for primers Pr2a and Pr2c and the probe previously described by Ibrahim et al. (10) were modified to suit the 5′ nuclease assay.

Bacterial strains and culture conditions. Bacterial strains that were evaluated are listed in Table 1. The *Y. enterocolitica* serotypes O:3 and O:9 were used as the reference strains in all optimization and sensitivity experiments. The *Y. enterocolitica* strains were enriched in peptone-sorbitol-bile broth (PSBB), incubated (35°C, 8 to 16 h), plated on cefsulodin-Irgasan-novobiocin (CIN) agar (Oxoid, Basingstoke, Hampshire, England), and incubated again (35°C, 18 to 24 h).

Development of 5′ nuclease assay probe for detection of virulent *Y. enterocolitica*. The primers (Prla, 5′ AATGCTGTC TTCATTTGGAGC 3′, and Prlb, 5′ ATCCCAATCACTACT GACTTC 3′) and probe sequences reported by Ibrahim et al. (10) were modified to suit the requirements of the 5′ nuclease assay. The fluorogenic probe (Yer-Prb FAM-CAAGCAAG CTTGTGATCCTCCG-TAMRA) specific to the *yst* gene (EMBL database, accession number X69218) was synthesized as previously described (2). The probe was an internal fluorogenic probe labeled with the reporter dye (FAM-6-carboxyfluorescein) at the 5′ end and a quencher (TAMRA-6-carboxytetramethylrhodamine) at the 3′ end (PE Applied Biosystems). Fluorescence was detected using a luminescence spectrometer (ABI Prism 7200 sequence detection system; PE Applied Biosystems) with a 96-well plate reader using the equation $\Delta RQ = RQ^{+} - RQ^{-}$ (2). A positive interpretation for pathogenic *Y. enterocolitica* was based on a threshold of four times the average ΔRQ value of no-template controls (25) from individual 96-well optical reaction plates (three no-template controls per plate). This enabled us to eliminate a lot of avirulent strains that fell in the positive interpretation range.

DNA extraction procedure. The DNA extraction procedure utilized the chelating properties of Chelex resin. Tenfold serial dilutions were made of PSBB cultures. Aliquots of 1 ml from each dilution were centrifuged (14,000 × *g*, 3 min), the supernatant was decanted carefully, and the pellet was resuspended in 200 μl of thoroughly mixed PrepMan sample preparation reagent (PE Applied Biosystems). The tubes were vortexed for 5 to 10 s or as long as required to resuspend the pellet, floated in boiling water (10 min), and chilled on ice (5 min). Then the tubes were centrifuged (14,000 × *g*, 3 min), and the supernatants were carefully transferred to new microcentrifuge tubes. A 2.5-μl aliquot of the supernatant served as the template for each PCR amplification in the 5′ nuclease assay.

PCR conditions. The PCR amplification conditions were different from those described by Ibrahim et al. (10). Briefly, 2.5 μl of sample containing the DNA template to be evaluated was added to 22.5 μl of PCR master mix (2.5 μl of 1× PCR buffer II [Perkin-Elmer], 2.5 to 6.0 mM $MgCl_2$, 300 nM concentrations of each primer [Pr2a and Pr2c], 200 μM deoxynucleoside triphosphate, 0.025 U of AmpliTaq DNA polymerase [Perkin-Elmer], 40 nM fluorogenic probe, and 22.5 μl of water) in disposable 96-well optical reaction plates (PE Applied Biosystems). Each set of reaction mixtures included a single row of wells of Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) for the autozero control and triplicate wells that were no-template controls (containing no *Y. enterocolitica* DNA templates). Each assay also included DNA from the avirulent strains of *Y. enterocolitica*, other species of *Yersinia*, and other bacteria listed in Table 1. All other bacteria were cultivated in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C for 15 h. The PCR had an initial denaturing step (95°C, 5 min) followed by 35 amplification cycles of a two-step PCR (94°C, 30 s; 44°C, 30 s, and 72°C, 30 s), with a final extension (72°C, 10 min) on a thermocycler (GeneAmp PCR system 9600; Perkin-Elmer).

Specificity studies with pure cultures. Specificity studies were performed utilizing DNA extracted from the *Yersinia*

TABLE 1. Bacterial strains evaluated and the ΔRQ values generated in PCR with the Pr2a and Pr2c primers and the Yer-prb fluorogenic probe

Bacterium evaluated	ΔRQ value[a]	Interpretation[b]
Y. enterocolitica (virulent serotypes)		
O:3[c]	29.26	Positive
O:9[c]	29.95	Positive
O:8[c]	29.65	Positive
O:18[c]	29.56	Positive
O:20[c]	29.13	Positive
O:21[c]	29.38	Positive
O:13[c]	29.39	Positive
Y. enterocolitica (avirulent serotypes)		
O:22[c]	3.36	Negative
O:22[d]	3.19	Negative
O:7[c]	2.89	Negative
O:7[e]	3.26	Negative
O:10[d]	3.05	Negative
O:10[e]	3.20	Negative
O:34[e]	3.33	Negative
Other species of *Yersinia*		
Y. aldovae[c]	2.24	Negative
Y. bercovieri[c]	2.05	Negative
Y. frederiksenii[c]	2.67	Negative
Y. intermediai[c]	2.71	Negative
Y. kristensenii[c]	1.93	Negative
Y. mollaretti[c]	2.29	Negative
Y. pseudotuberculosis[f]	1.85	Negative
Y. ruckeri[c]	2.82	Negative
Other bacteria		
Citrobacter freudinii[f]	2.24	Negative
E. coli[g]	1.26	Negative
E. coli O157:H7[g]	1.49	Negative
Flavobacterium spp.[g]	1.24	Negative
Salmonella enterica serotype chloleraesius[g]	0.81	Negative
S. enterica serotype Typhimurium[g]	1.54	Negative

[a] Determined with ~10^7 CFU/ml for each strain.
[b] A positive score is assigned when the ΔRQ is greater than the ΔRQ threshold, based on a fourfold average value of the no-template controls.
[c] From the National Animal Disease Center, United States Department of Agriculture. Ames, Iowa.
[d] From the Centers for Disease Control and Prevention, Atlanta, Ga.
[e] From the Food Microbiology Culture Collection, Kansas State University, Manhattan.
[f] From M. M. Chengappa, Veterinary Diagnostic Laboratory, Kansas State University.
[g] From R. D. Oberst, Food Animal Health and Management Center, Kansas State University.

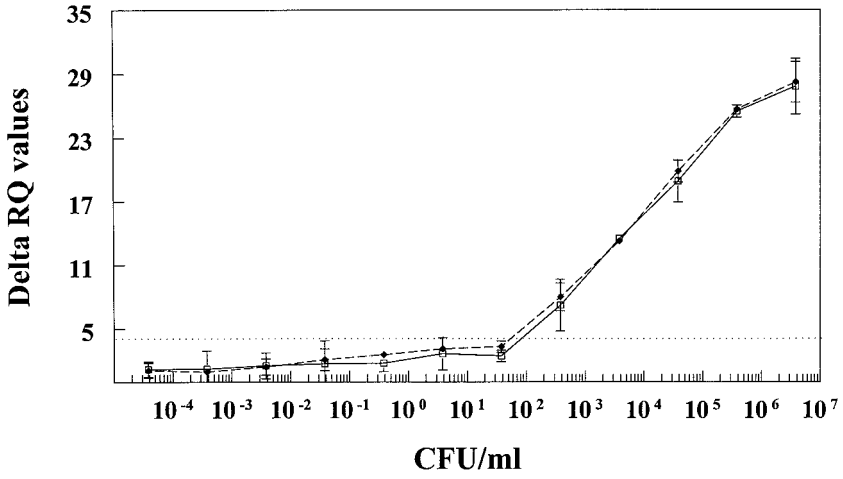

FIG. 1. Sensitivity of the fluorogenic 5′ nuclease assay for detecting pathogenic *Y. enterocolitica* in disposable 96-well optical reaction plates (PE Applied Biosystems). Tenfold dilutions of *Y. enterocolitica* serotypes O:3 (□) and O:9 (◆) were made in PSBB in triplicate. One-milliliter aliquots from each dilution were subjected to DNA extraction using the PrepMan method, and 2.5 μl of the recovered DNA solution was PCR-amplified using the Pr2a and Pr2c primers in the presence of the Yer-prb fluorogenic probe. Detection and analysis were completed with the ABI Prism sequence detection system. All amplification and detection reactions were completed in 96-well optical reaction plates. The average ΔRQ values for each dilution were plotted against the average number of CFU per milliliter as determined by plating each dilution on CIN agar. The adjusted ΔRQ threshold value was calculated to be 4.44 (dashed horizontal line). Error bars indicate the standard deviations of the means.

species and serotypes and other bacteria (Table 1). Pure cultures of virulent and avirulent *Y. enterocolitica* were grown in PSBB (35°C, 8 to 16 h) and the other *Yersinia* spp. and bacteria were grown in brain heart infusion broth (37°C, 8 to 16 h). Sensitivity studies utilizing pure cultures of *Y. enterocolitica* serotypes O:3 and O:9 also were performed to identify the lower detection limit of the 5′ nuclease assay. The cultures were grown overnight, serially diluted (10-fold), and enumerated on CIN plates. Then DNA was extracted using PrepMan sample preparation reagent and was run through the 5′ nuclease assay. The experiment was replicated three times. All the virulent *Y. enterocolitica* strains gave a positive reaction with the ΔRQ values above the threshold of 4.44 (four times the average ΔRQ of the no-template controls), whereas the avirulent *Y. enterocolitica*, *Yersinia* spp., and other bacterial species gave a "no" interpretation with ΔRQ values below the threshold. Similar to the PCR results described by Ibrahim et al. (10), the assay was found to be 100% specific in identifying the pathogenic strains of *Y. enterocolitica*.

Sensitivity studies with pure cultures and spiked ground pork samples. Sensitivity studies were performed on pure cultures of virulent *Y. enterocolitica* serotypes (O:3 and O:9) to test the lower detection limit of the fluorogenic 5′ nuclease assay. When pure cultures of O:3 and O:9 grown in PSBB for 8 to 16 h were enumerated and run through the DNA extraction and fluorogenic 5′ nuclease assay, the ΔRQ values were greater than the detection threshold of 4.44 when $\geq 10^2$ CFU/ml were present (Fig. 1). Dividing the lowest dilution of the culture that gave a positive reaction by the approximate final volume of the DNA extraction (CFU per microliter) gave a lower detection limit of ≥10 CFU per PCR. In our assay, the lowest detection limit was 9.4 CFU/PCR. This was followed by DNA extraction, PCR, and fluorescence detection. This experiment was repeated three times.

Ground pork obtained from local grocery stores in Manhattan, Kans., was confirmed to be culture negative for *Y. enterocolitica*. Then 25-g samples were spiked with 10^3 CFU of *Y. enterocolitica* serotypes O:3 and O:9, enriched in 225 ml of PSBB, and incubated at 35°C for 12 h. The sensitivity of the 5′ nuclease assay in identifying virulent *Y. enterocolitica* in spiked ground pork samples was found to be approximately 10^3 CFU/g with a threshold ΔRQ value of 6.68 and above (Fig. 2). The sensitivity for spiked samples was calculated to be $\geq 10^2$ CFU/PCR. In our assay, the lowest detection limit per PCR was 36 CFU for spiked samples.

Unknown sample study. A hundred samples of ground pork purchased at three different grocery stores in Manhattan, Kans., were enriched in PSBB (25 g of sample in 225 ml of PSBB) for 18 h and then tested for the presence of virulent *Y. enterocolitica* by conventional culture methods, two virulence tests, and the 5′ nuclease assay. One loopful of the preenriched sample was streaked on CIN agar plates which were incubated for 24 to 48 h at 35°C. After 48 h, suspected *Y. enterocolitica* colonies were tested using the API 20E system (bioMerieux, Hazelwood, Mo.). All the colonies that tested positive for *Y. enterocolitica* by the API 20E System were further tested for virulence by autoagglutination and crystal violet binding tests (29). The conventional methods identified *Y. enterocolitica* in 45 of the 100 samples of ground pork. Out of these 45 samples, only 35 were identified as being virulent by 5′ nuclease assay. These 35 isolates were also the only samples that were positive for virulence by the crystal violet binding and autoagglutination tests.

The described fluorogenic 5′ nuclease assay was successful in detecting virulent strains of *Y. enterocolitica* within 5 h after an 18 h enrichment. Positive interpretations were obtained for all reference strains of virulent *Y. enterocolitica* that were evaluated for pure cultures of serotypes O:3 and O:9 and for ground pork samples spiked with serotypes O:3 and O:9. This automated amplification and detection procedure could be a reliable rapid-screening method for detecting virulent *Y. enterocolitica* DNA.

Sensitivity studies involving pure cultures and spiked ground pork enrichments demonstrated that the assay was reliably sensitive, with lower detection limits of 10^2 to 10^3 CFU/ml or CFU/g under both conditions. Based on these data, a ΔRQ value of 4.44 and above was considered positive for the presence of virulent *Y. enterocolitica* in pure cultures, and a ΔRQ

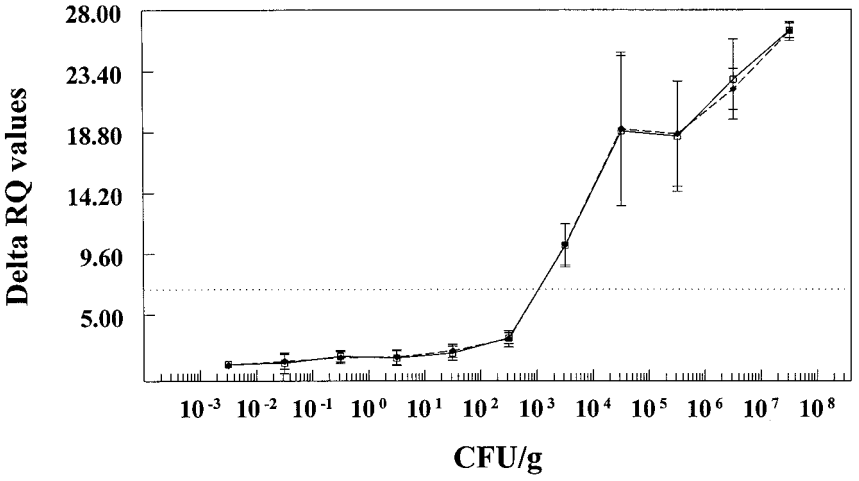

FIG. 2. Sensitivity of the fluorogenic 5′ nuclease assay for detecting pathogenic *Y. enterocolitica* in spiked ground pork samples. Tenfold dilutions of *Y. enterocolitica* serotypes O:3 (□) and O:9 (◆) in PSBB in triplicate after enrichment at 35°C for 12 h. Aliquots (1 ml) were collected for DNA recovery using the PrepMan extraction method, and 2.5 μl of the recovered DNA solution was amplified with the Pr2a and Pr2c primers in the presence of the Yer-prb fluorogenic probe. Detection and analysis were completed with the ABI Prism sequence detection system. All amplification and detection reactions were completed in 96-well optical reaction plates. The average ΔRQ values determined from DNA recovered from both serotypes were plotted against the average number of CFU per milliliter determined by plating each ground pork dilution on CIN agar. The ΔRQ threshold value at four times the average of no-template controls was calculated to be 6.68 (dashed horizontal line). Error bars indicate the standard deviations of the means.

value of 6.68 and above was considered positive for spiked ground pork samples.

The virulent *Y. enterocolitica* strains among the 28 bacterial cultures tested for specificity gave positive interpretations, with ΔRQ values above 6.68 in all three replications. Therefore, this assay was found to be 100% specific for detecting pathogenic strains of *Y. enterocolitica*.

The unknown sample study was carried out to evaluate the use of the 5′ nuclease assay in actual food testing. The results of this assay were consistent with those of the genotypic virulence tests and those of cultural methods. Because not all strains of *Y. enterocolitica* are virulent, virulence tests are needed in order to determine if the food sample harbors a virulent strain. Most of these virulence tests are time consuming and laborious and require 24 to 30 h of incubation after selective enrichment and isolation, which require 2 to 3 days. Identifying virulent *Y. enterocolitica* in food or clinical samples by cultural methods requires 4 to 5 days, which adds even more time and expense to the detection procedure.

There are considerable difficulties associated with the isolation of *Y. enterocolitica* from foods (11, 15). Most methods require time-consuming enrichments to achieve optimal isolation. Moreover, no currently available method allows optimal recovery of all virulent serotypes. However, the 5′ nuclease assay developed by us requires only 5 h of processing (DNA extraction, PCR amplification, and detection) after overnight enrichment. The problem with all selective media described so far, including CIN, is that they provide inadequate differentiation between virulent and avirulent *Y. enterocolitica* (11). The avirulent variants are common in many foods, and their colony morphology makes them difficult to distinguish from the virulent strains (15).

Other molecular systems for detecting pathogenic *Y. enterocolitica*, such as multiplex riboprobes (7) that used a pool of RNA probes specific for various chromosomal and plasmid-borne virulence genes, oligonucleotide probes (11) for the detection of virulent *Y. enterocolitica* and PCR (31) require additional processing steps that increase the analysis time. The main advantage of this 5′ nuclease assay is the rapidity with which it screens for the presence of virulent *Y. enterocolitica*. The detection system uses a 96-well fluorescence plate reader with optical tubes and caps, so 96 samples can be analyzed at a time. Plate readings do not require tubes to be opened after amplification, so the potential for carryover is reduced. Therefore, this 5′ nuclease assay is more applicable than other systems for the detection of pathogenic *Y. enterocolitica* in food production and processing facilities.

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