Evaluation of a 5’-Nuclease (TaqMan) Assay for the Detection of Virulent Strains of *Yersinia enterocolitica* in Raw Meat and Tofu Samples

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ABSTRACT

Culture methods for detecting virulent *Yersinia enterocolitica* require selective enrichment and a series of confirmatory tests that are time-consuming, costly, and laborious. The objective of this study was to evaluate a fluorogenic 5’-nuclease assay for detecting the enterotoxin *yst* gene of virulent *Y. enterocolitica* in pure cultures, inoculated ground pork samples, and naturally contaminated food samples. These results were then compared with “gold standard” methods recommended by the U.S. Food and Drug Administration in the *Bacteriological Analytical Manual* for detecting pathogenic *Y. enterocolitica*. The 5’-nuclease assay was able to identify the organism in 100% of the repetitions when 10^5 CFU/ml or more organisms were present in pure cultures and 10^3 CFU/g or more organisms were present in ground pork. Similar recovery efficiency on cefsulodin-irgasan-novobiocin (CIN) agar plates was only evident when 10^5 CFU/ml or more organisms were present in pure culture and 10^3 CFU/g or more organisms were present in inoculated ground pork. The 5’-nuclease assay indicated a contamination rate of 35.5% (94/265) in various meats and tofu, whereas the CIN plating method indicated a contamination rate of 28.3% (75/265). This resulted in 100% sensitivity and 64.5% specificity for the 5’-nuclease assay when compared with the standard culture recovery method. Only 75% (60/80) of the *Yersinia* spp. isolated on CIN was identified as containing a virulence plasmid by autoagglutination and crystal violet binding tests. These results indicate that the true rate of contamination of virulent *Y. enterocolitica* in pork and other processed meats and foods is being underestimated using current detection methods. This study demonstrates the potential of the 5’-nuclease assay for rapidly and specifically detecting virulent *Y. enterocolitica* in processed foods with the added advantage of being an automated detection system with high-throughput capability.

*Yersinia enterocolitica* is widely recognized as an important foodborne pathogen of humans. It is an invasive enteropathogen that most commonly causes enterocolitis in humans (1). This organism is found free living in the environment or in association with a host. There is strong evidence that food, especially pork, and unchlorinated water are major sources of infection (16). Swine have been implicated as the principal reservoir for human virulent *Y. enterocolitica* (1). Virulence in *Y. enterocolitica* results from a complex interplay between a series of plasmid and chromosomal genes (8, 29). The chromosomal *ysh* gene encoding a low-molecular-weight, heat-stable enterotoxin is confined to the virulent bioserotypes of *Y. enterocolitica* and, hence, is a useful marker of virulence (14, 15). Conventional culture methods for detecting virulent *Y. enterocolitica* require a series of biochemical and serological tests that are time-consuming and laborious (26, 33). Several investigators have developed immunological and DNA-based methods for the detection of virulent *Y. enterocolitica* (11, 14, 15, 17, 19, 20). One of the most promising methods for detecting virulent *Y. enterocolitica* is based on the polymerase chain reaction (PCR), which has relatively high sensitivity and specificity. However, detection of the PCR product involves electrophoresis, which is time-consuming and also uses ethidium bromide to stain agarose gels. Ethidium bromide is a mutagen and may not be appropriate for routine use in food-monitoring laboratories. Fluorogenic 5’-nuclease assays have been described that allowed the automated PCR amplification, detection, and analysis of *Salmonella* spp. (6, 18, 25), *Listeria monocytogenes* (2, 3), *Escherichia coli* O157: H7 (28), and Shiga-like toxin genes (12, 34) in various foods. The 5’-nuclease assay exploits the 5’ 3’ activity of *Thermus aquaticus* DNA polymerase (13, 21) to hydrolyze an internal TaqMan probe labeled with two fluorescent dyes (23). The probe is designed to hybridize to an internal region of the targeted sequence. For the intact probe, the fluorescence from a reporter dye is suppressed by a quencher dye because of 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 on a fluorometer. Because the increase in fluorogenic reporter signal is a direct consequence of a successful PCR, this can be used in the detection of specific DNA sequenc-
es. The fluorometric data can be read automatically and interpreted using a 96-sample format and presented as a positive or negative conclusion for the presence or absence of the predicted PCR product.

In this study we describe evaluations of a 5′-nuclease assay for the automated, PCR-based amplification, detection, and analysis of the heat-stable enterotoxin yst gene present in virulent Y. enterocolitica. Evaluations of the 5′-nuclease assay were compared with standard methods for recovering and identifying virulent Y. enterocolitica in pure cultures, seeded ground pork, and raw meat and tofu samples purchased from local grocery stores.

**MATERIALS AND METHODS**

**Sensitivity and specificity.** The sensitivity and specificity of the 5′-nuclease assay was calculated in 2 by 2 analyses (24) in comparative studies using cultural isolation on ceftazidime-irgasan-novobiocin (CIN) agar and several pathogenicity testing procedures (autoagglutination and crystal violet [CV] binding tests) as recommended by the U.S. Food and Drug Administration *Bacteriological Analytical Manual* (33). The combined conclusions of the recovery and pathogenicity testing procedures were subsequently referred to as the “gold standard” virulent *Y. enterocolitica* detection method.

**Sensitivity of the assay with pure culture and inoculated ground pork.** Sensitivity studies using pure cultures of *Y. enterocolitica* serotype O:3 (accession no. 5563; National Animal Disease Center, Ames, Iowa) were performed to identify the detection limit of the 5′-nuclease assay. The culture was inoculated in 10 ml of peptone sorbitol bile broth (PSBB) (33), incubated (35°C, 16 h), and then serially diluted (10-fold) in triplicate and enumerated in duplicate with 0.1 ml of PSBB streaked on CIN agar plates (Oxoid, Hampshire, England) and incubated (35°C, 18 to 24 h). The DNA from 1.0 ml of PSBB was recovered and used in triplicate 5′-nuclease assays.

For the inoculated ground pork study, 25-g samples (previously confirmed to be culturally negative for *Y. enterocolitica* by CIN plating) were mixed with 225 ml of PSBB in sterile stomacher bags, inoculated with approximately 10⁵ CFU of *Y. enterocolitica* serotype O:3, and pummeled for 2 min in a stomacher 400 Lab Blender (Seward, London, England). The bags were then incubated (35°C, 18 h), with a final extension (72°C, 10 min) on a thermocycler (GeneAmp PCR System 9600, Applied Biosystems). Fluorescence was detected with a luminescence spectrometer (ABI Prism 7200 Sequence Detection System, Applied Biosystems) using a double-labeled fluorogenic probe (FAM-CAAGCAAGCTTGTGATCCTCGC-CG-TAMRA) in 5′-nuclease assays specific for detecting the yst gene of virulent *Y. enterocolitica* as recently reported (32). Briefly, the oligonucleotide probes were synthesized with the reporter dye (FAM-6-carboxy-fluorescein) at the 5′ end and the quencher dye (TAMRA-6-carboxytetramethylrhodamine) at the 3′ end (Applied Biosystems, Foster City, Calif.) (2).

**Autoagglutination test.** The cultures that were identified as *Y. enterocolitica* on CIN and confirmed by biochemical evaluations were inoculated into tubes containing methyl red Voges Proskauer broth (Difco Laboratories, Detroit, Mich.) and incubated (24 h, 35 to 37°C). Following incubation the tubes were observed for agglutination (clumping) along the walls and/or the bottom of the tube with clear supernatant. If this agglutination profile was present, the isolate was recorded as being autoagglutination positive, whereas a lack of agglutination or any other pattern of agglutination at this temperature was considered negative (33).

**CV binding test.** The CIN culture-positive isolates of *Y. enterocolitica* that were identified to be positive for autoagglutination were grown in brain heart infusion broth (Difco) with shaking (22 to 26°C, 18 h). The cultures were diluted to 1,000 cells/ml in physiological saline, and 0.1 ml of the culture was spread on each of two brain heart infusion agar plates. One plate was incubated at 25°C for 30 h and the other at 37°C for 30 h. Each plate was flooded with 8 ml of CV solution (85 μg/ml) for 2 min, and the excess was removed. Virulent *Y. enterocolitica* cultures bind CV, and colonies grown at 37°C appear purple, but colonies grown at 25°C are not purple (33). Conversely, avirulent *Y. enterocolitica* cultures do not bind CV and colonies do not appear purple.

**Fluorogenic 5′-nuclease assay for detection of virulent Y. enterocolitica.** The PCR primers (Pr1a 5′ AATGCTGTCTTCA-TTTGGAGC 3′ and Pr1b 5′ ATCCCAATCRAFTGCTTC 3′) described by Ibrahim et al. (14) were used with a double-labeled fluorogenic probe (FAM-CAAGCAAGCTTGTGATCCTCCG-TAMRA) in 5′-nuclease assays specific for detecting the yst gene of virulent *Y. enterocolitica* as recently reported (32). Briefly, the oligonucleotide probes were synthesized with the reporter dye (FAM-6-carboxy-fluorescein) at the 5′ end and the quencher dye (TAMRA-6-carboxytetramethylrhodamine) at the 3′ end (Applied Biosystems, Foster City, Calif.) (2).

**DNA extraction procedure.** One-milliliter aliquots of samples in PSBB were centrifuged (14,000 x g, 3 min) and the supernatant was decanted. The pellet was resuspended in 200 μl of thoroughly mixed PrepMan Sample Preparation Reagent (Applied Biosystems) and vortexed for approximately 5 to 10 s or as long as required to resuspend the pellet, heated in boiling water (10 min), and chilled on ice (5 min). The tubes were then centrifuged (14,000 x g, 3 min), and the supernatants carefully transferred to new microcentrifuge tubes for storage at −20°C or PCR amplification.

**PCR conditions.** Briefly, 2.5 μl of each sample containing the DNA template to be evaluated was added to 22.5 μl of PCR master mix (2.5 μl of 1 X PCR buffer II [Applied Biosystems]; 2.5 to 6.0 mM of MgCl₂; 300 nM of each primer [Pr1a and Pr1b] (32); 200 μM of dNTP; 0.025 U of AmpliTaq DNA polymerase [PE Biosystems]; 20 to 50 nM of fluorogenic probe; and 22.5 μl of water) in disposable 96-well optical reaction plates (Applied Biosystems). Each set of reactions included six wells of TE buffer (10 mM of Tris-HCl, pH 8.0; 1 mM of EDTA) that served as the autozero control and three wells that were no-template controls (containing no *Y. enterocolitica* DNA templates). The PCR had an initial denaturation 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, Applied Biosystems). Fluorescence was detected with a luminescence spectrometer (ABI Prism 7200 Sequence Detection System, Applied Biosystems) us-
TABLE 1. Sensitivity of 5'-nuclease assay and culture methods to detect pure cultures of virulent Y. enterocolitica serotype O:3

<table>
<thead>
<tr>
<th>Concentration (CFU/ml)</th>
<th>Recovered by culture method</th>
<th>5'-nuclease assay positive</th>
<th>Average ΔRQ values (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.8 × 10⁶</td>
<td>6/6</td>
<td>3/3</td>
<td>28.2 (26.1–29.9)</td>
</tr>
<tr>
<td>3.6 × 10⁵</td>
<td>6/6</td>
<td>3/3</td>
<td>25.6 (25.5–26.1)</td>
</tr>
<tr>
<td>2.4 × 10⁴</td>
<td>5/6</td>
<td>3/3</td>
<td>19.9 (19.0–21.0)</td>
</tr>
<tr>
<td>3.1 × 10³</td>
<td>3/6</td>
<td>3/3</td>
<td>13.3 (13.0–13.7)</td>
</tr>
<tr>
<td>2.5 × 10²</td>
<td>2/6</td>
<td>3/3</td>
<td>8.0 (6.5–8.9)</td>
</tr>
<tr>
<td>≤10</td>
<td>0/6</td>
<td>0/3</td>
<td>3.3 (2.9–3.9)</td>
</tr>
<tr>
<td>1</td>
<td>0/6</td>
<td>0/3</td>
<td>3.1 (2.8–3.5)</td>
</tr>
<tr>
<td>≤1</td>
<td>0/6</td>
<td>0/3</td>
<td>2.5 (2.2–2.9)</td>
</tr>
</tbody>
</table>

a Average of three replications as determined by plating on cef-sulodin-irgasan-novobiocin (CIN) agar.
b Three replications of each dilution, plated in duplicate. Based on number identified as positive for Y. enterocolitica number examined by plating on CIN agar and biochemical confirmation.
c Three replications of each dilution evaluated with 5'-nuclease assay. Based on number identified as positive for virulent Y. enterocolitica number examined. All 5'-nuclease assay-positive samples had ΔRQ values of more than 4.44.

RESULTS

Sensitivity to detect pure cultures of Y. enterocolitica serotype O:3. A positive indication for identifying the yst gene of virulent Y. enterocolitica in pure cultures with the 5'-nuclease assay was based on exceeding a threshold of four times the average ΔRQ value of three no-template controls per plate from individual 96-well optical reaction plates (32).

Sensitivity to detect virulent Y. enterocolitica in inoculated ground pork. The threshold level of the 5'-nuclease assay to detect virulent Y. enterocolitica in inoculated ground pork samples was indicated when ΔRQ values were more than 6.68 or when a detection level of 10⁵ CFU/g or more was present (Table 2). At concentrations of 10⁶ CFU/g or more, 100% agreement was demonstrated in the conclusions of all three replicates of the 5'-nuclease assay (Table 2). This contrasted with the CIN plating method, which was able to identify Y. enterocolitica in all replications only when 10⁵ CFU/g or more organisms were present (Table 2). Recovery of lower numbers of organisms (between 10³ and 10⁴ CFU/g) by CIN plating was not possible in all replications. Virulence determination of culturally identified isolates on CIN plates, by autoagglutination and CV binding, was not attempted in the inoculated ground pork samples.

Detection of virulent Y. enterocolitica in foods. Plating food samples on CIN agar indicated that 40 ground pork samples, 28 ground beef samples, 7 porcine tongue samples, and 0 tofu samples were contaminated with Y. enterocolitica (Table 3). Confirmation of the virulence of these isolates by autoagglutination and CV binding demonstrated that only 60 (86%) of the 70 CIN isolates were positive for both assays, but all 70 isolates were identified as being virulent Y. enterocolitica by the 5'-nuclease assay. In addition, the 5'-nuclease assay identified 19 samples as containing virulent Y. enterocolitica that were culture negative by CIN plating. These conclusions were confirmed when restreaking of these 19 samples resulted in no red or pink colonies being identified on CIN plates. In comparison with the conclusions of the cultural recovery and virulence detection methods as the “gold standard” detection procedure, the 5'-nuclease assay was shown to have a sensitivity of 100% and a specificity of 64.5% in food samples recovered from local stores.
that currently used detection methods are incapable of detecting low numbers of virulent in the oral cavity or throat tissues of pigs. Based on these findings we conclude that the true rate of occurrence of virulent identified by plating on cefsulodin-irgasan-novobiocin (CIN) agar and API 20 E on red to pink colonies. In contrast, Logue et al. identified Y. enterocolitica identified by autoagglutination and crystal violet binding. Not detected by CIN and API 20E methods. Detected by CIN and API 20E but not confirmed for virulence by autoagglutination and crystal violet binding. Could not be evaluated due to the absence of any samples positive by culture methods.

**TABLE 3. Comparison of different methods to detect virulent Y. enterocolitica in food samples**

<table>
<thead>
<tr>
<th>Sample type</th>
<th>No. of samples</th>
<th>Culture method</th>
<th>Virulence tests</th>
<th>5'-nuclease assay</th>
<th>Culture negative and 5'-nuclease assay positive</th>
<th>Virulence negative and 5'-nuclease assay positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground pork</td>
<td>100</td>
<td>40</td>
<td>32</td>
<td>47</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Ground beef</td>
<td>100</td>
<td>28</td>
<td>23</td>
<td>31</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Tofu</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>NA</td>
</tr>
<tr>
<td>Porcine tongues</td>
<td>15</td>
<td>7</td>
<td>5</td>
<td>10</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>265</td>
<td>75</td>
<td>60</td>
<td>94</td>
<td>19</td>
<td>15</td>
</tr>
</tbody>
</table>

* Samples purchased from local grocery stores in Manhattan, Kan.

**DISCUSSION**

The prevalence of virulent Y. enterocolitica in processed pork is controversial. Durisin et al. (10) reported the detection of virulent Y. enterocolitica in 14% of the processed pork samples using DNA hybridizations using a yst gene probe. In contrast, Logue et al. (22) reported identifying contamination in 70% of the raw pork obtained at retail outlets in Ireland using a postenrichment alkali treatment to improve isolation of Yersinia spp. We identified virulent Y. enterocolitica in 47% of the ground pork samples using a 5'-nuclease assay following selective enrichment in PSBB but in only 32% of the ground pork samples using PSBB-CIN culture recovery method and consensus between two virulence assays (autoagglutination and CV binding tests). We also identified virulent Y. enterocolitica in 66.6 and 33.3% of the pork tongues evaluated using either the 5'-nuclease assay or a combined culture and virulence method, respectively. Previous reports involving conventional culture and virulence methods (7, 27) have also demonstrated high occurrence of virulent Y. enterocolitica in the oral cavity or throat tissues of pigs.

Based on these findings we conclude that the true rate of occurrence of virulent Y. enterocolitica in pork and other processed meats and foods is being underestimated using current methods for recovery and virulence characterization. We agree with the conclusion of Durisin et al. (10) that currently used detection methods are incapable of detecting low numbers of virulent Y. enterocolitica in processed pork products at the point of retail sale. The 5'-nuclease assay results with ground pork and porcine tongues are similar to what Nesbakken (27) found in assessing the high rates of Y. enterocolitica contamination on hog carcasses. This would indicate that current slaughter and processing procedures for reducing virulent Y. enterocolitica in pork and beef are not successful. Our data would substantiate Nesbakken’s (27) concern that because of the high rate of occurrence of Y. enterocolitica in the oral cavity of pigs, there is a good likelihood that current slaughter and processing conditions might contribute to an increased rate of carcass contamination. Increased carcass contamination could then lead to a higher frequency of contamination in subsequent processing.

False-negative results are unacceptable conclusions in the microbiological assessment of pathogens in foods. In this study, the conclusions of the 5'-nuclease assay indicated that some food samples contained virulent Y. enterocolitica that were not detected by the cultural or virulence methods. Several reasons might be responsible for these findings. First is the insensitivity of the CIN agar recovery method. This was demonstrated in comparative studies with pure cultures and inoculated ground pork. Similarly, the 5'-nuclease assay identified 19 food samples that contained the yst gene, but no organisms were recovered on CIN agar. Clinical strains of Yersinia, including those of serotype O:8, which are commonly associated with foodborne outbreaks of yersiniosis, have been shown to be sensitive to selective agents in CIN agar (9). It is also important to consider the possibility that low numbers of some healthy or injured and stressed cells in some foods might require longer enrichment times to allow recovery of the organisms to threshold levels by culture methods (31). The inability to recover virulent Y. enterocolitica from tofu, but the identification of the yst gene in 12% (6/50) of samples examined, indicates that further research will be required to assess the importance of different enrichment processes as a component of the overall 5'-nuclease detection process. Regardless, insensitivity of the CIN recovery method was demonstrated with pure cultures and in spiked pork samples and resulted in false-negative conclusions in some replicates when the concentrations of organisms were less than 10³ CFU/ml in pure cultures and less than 10⁶ CFU/gm in spiked ground pork. The limiting threshold of the described procedure for detecting virulent Y. enterocolitica with the 5'-nuclease assay was 10² CFU/ml in pure cultures and 10³ CFU/gm in spiked ground pork.

The culture recovery method of enrichment and plating on CIN agar lacks the ability to differentiate between virulent and avirulent Y. enterocolitica isolates. Therefore, additional tests are currently required to assess their virulence. Autoagglutination and CV binding assays are presumptive
screening tests based on expression of phenotypes that are conferred on the bacteria by virulence plasmids (33). These plasmids have been shown to be unstable and can be lost during routine cultural procedures (4, 5, 8, 33). This has necessitated the practice of maintaining temperatures of 30°C or less for incubation and rapidly freezing presumptive-positive Yersinia cultures to ensure that lengthy periods of cultivation or temperatures of more than 30°C do not result in the spontaneous loss of virulence (33). Loss of these genes from previously characterized virulent isolates of Y. enterocolitica does occur and can result in PCR-based false-negative conclusions about the virulence of an isolate subsequent to its initial recovery and classification (35).

It is possible that instability or alterations in expression of the virulence plasmid could have been involved in the 15 food samples that were identified as being virulent isolates by the 5′-nuclease assay but were not identified as virulent in the consensus conclusion of the autoagglutination and CV binding assays. The potential for carryover contamination in the 5′-nuclease assay could also result in false-positive conclusions. Although a possibility, this was not indicated in the earlier pure culture studies or inoculated sample studies or in any of the controls or adjacent samples on the disposable 96-well optical reaction plates (data not shown). Possible reasons for these results might be that the 5′-nuclease assay was completed before the completion of the autoagglutination and CV binding assays. Because each of these assays involved additional cultivation procedures following the completion of the 5′-nuclease assay, the autoagglutination tests were completed at temperatures of more than 30°C (35 to 37°C). Either of these practices could result in loss or instability of the virulence plasmid and inaccurate conclusions about the virulence of an isolate (33). Additional research will be needed to incorporate adequate 5′-nuclease internal positive controls (TagMan Internal Positive Control, Applied Biosystems) in sample processing procedures and in the 5′-nuclease assay to ensure that the potential for 5′-nuclease false-negative conclusions based on the presence of inhibitors and inadequate DNA recovery is minimized.

Advances in detecting virulent strains of Y. enterocolitica have recently been demonstrated using methods that rely on nucleic acid–based hybridization techniques or mouse infectivity assays, but these procedures require lengthy processing steps (11, 17, 19, 23). These assays can take an additional 24 to 30 h for DNA hybridization or more than a week after mouse inoculations to determine virulence of the isolate. The application of PCR has also been successful toward detecting virulent Y. enterocolitica, but this process also requires time-consuming, laborious, and technically demanding post-PCR processing steps to identify and analyze the specificity of the amplified PCR products (15). The results of this study indicate the potential of the 5′-nuclease assay for rapidly and specifically detecting virulent Y. enterocolitica in pork and processed meats and other foods. The 5′-nuclease assay demonstrated in this study had the added advantage of being an automated detection process with a high-throughput capability and adaptability. We believe that future improvements in the current 5′-nuclease assay that focus on incorporating positive internal controls throughout the DNA recovery process and amplification steps and increasing the automation of the entire process will improve the assay’s usefulness as a rapid detection method for virulent Y. enterocolitica in pork, other meat products, and foods.

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