Comparison of a Multiplex and 5' Nuclease PCR Assays for the Rapid Detection of Pathogenic *Yersinia enterocolitica* in Swine and Pork Products

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ASL-R1705

Summary and Implications

Bacteriological culture methods were compared with PCR based protocols (multiplex PCR and TaqMan assay) for the rapid detection of pathogenic *Yersinia enterocolitica* (YE) in market weight hogs and pork products. The prevalence of YE was compared in lairaged hogs (n=150) versus hogs transported directly to the farm (n=150). By bacteriological culture, YE was not detected in any of the hog tissues tested but was detected by multiplex PCR and TaqMan assay. We also screened ground pork and chitterlings for the presence of YE. By standard culture, YE was detected in chitterlings (8%). By multiplex PCR, YE was identified in ground pork (10%) and chitterlings (27%). TaqMan assay identified YE in ground pork (44%) and chitterlings (79%).

Introduction

Yersinia enterocolitica (YE) is an important causative agent of many gastrointestinal diseases in animals and humans (2). As an enteropathogen, it is frequently involved in human enterocolitis, severe abdominal pain mimicking appendicitis, mesenteric lymphadenitis, and systemic infection (6). There is a strong evidence that pigs and pork products are the major reservoirs for human infection with YE (14). YE has been repeatedly isolated from tonsils, tongue, cheek meat, and intestinal tract of healthy pigs (7,9).

The use of polymerase chain reaction (PCR) to identify pathogenic YE has been reported (8,11). A multiplex PCR targets two genes: the chromosomally encoded *ail* gene (13) and the plasmid-borne virulence *yadA* gene (3). The protocol for this PCR assay utilizes lengthy enrichment steps, and required laborious gel-based analysis of PCR amplified products.

The 5' nuclease PCR assay (TaqMan assay) is a new PCR sequence detection system that uses the 5' nuclease activity of *Taq* DNA polymerase and detects the PCR amplified products by hybridization and cleavage of a double-labeled fluorogenic probe during the amplification (Figure 1) (12). This method has been used to detect *Listeria monocytogenes* (1), *Escherichia coli* O157:H7 (15) and *Salmonella spp.* (5).

Our objectives were (1) to compare bacteriological culture with PCR-based protocols (multiplex PCR and TaqMan assay) for detecting pathogenic strains of YE, and (2) to use these assays to screen hogs and pork products for YE. We also used these assays to determine if lairage of hogs increases the fecal shedding, lymph node, and carcass prevalence of YE. YE prevalence was compared in lairaged hogs (n=150) versus hogs transported directly from the farm to slaughter (n=150). We also screened ground pork and chitterlings to determine the percentage of YE contamination in these processed pork products.

Materials and Methods

Bacterial strains. YE strains were obtained from the reference collection at the National Animal Disease Center (NADC). Strain NADC 5561 was used as a positive control in multiplex PCR and TaqMan assay. Non-YE strains were used as negative controls: *Y. aldovae*, NADC 5612; *Y. bercovieri*, NADC 5615; *Y. frederiksenii*, NADC 5617; and *Y. kristensenii*, NADC 5622.

Samples and sample preparation. A total of 300 market weight hogs (30 each time) were screened for the presence of YE. Thirty hogs were randomly selected at the farm and feces and tonsil scrapings were collected. Thirty hogs were divided into two groups; fifteen hogs were brought to NADC and held overnight without feed and the following day were taken to the abattoir; the remaining 15 hogs were transported directly to the abattoir. At slaughter, tonsils, ileocecal, ventral thoracic, superficial inguinal lymph nodes, carcass swabs, and cecal and rectal contents were collected in sterile whirl paks and transported to the laboratory on ice. Tonsils and lymph nodes were homogenized with 25 ml of sterile buffered peptone water (pH 7.4) in a stomacher (1 min). Tissue homogenates (1 ml) and cecal and rectal contents (1 g) were inoculated each, into 9 ml of Irgasan, ticarcillin, potassium chlorate (ITC) enrichment broth. Swab samples were taken from the ham and ventral surface of the carcass. The swabs (3 cm × 1cm × 0.5 cm) were placed in 9 ml of ITC.

Processed pork products. Ground pork and chitterlings were procured from the packing plant the same day of slaughter. Seven trials were conducted (50 samples per trial). Pork product (25 g) was inoculated into 225 ml of ITC.

Enrichment. ITC was used as a selective enrichment medium (16). Previous studies conducted in our laboratory showed that ITC enrichment was superior compared with MTSB and PBS enrichments (17). After enrichment (room temperature for 48 h), 100 µl aliquot was plated to CIN agar (Oxoid, cat. # CM 653), incubated overnight at 30°C, and examined for typical bull's-eye colonies.

Genomic DNA extraction. Template DNA was extracted with guanidine extraction procedure based on the DNA purification protocol by silica particles (4). In short, a 1 ml aliquot of each ITC enrichment sample was pelleted (1 min, 14,000 rpm); the pellet was resuspended in 0. 5 ml of DNA binding solution (1% diatomaceous earth in 6 M guanidine hydrochloride) and frozen (–80°C) to lyse cells. The mixture was thawed, diatoms pelleted, and washed twice with 70% ethanol. DNA was eluted from the diatom pellet by resuspending it in 50 _l of sterile water, heating at 65°C for 10 min, pelleting the diatoms (1 min, 14,000 rpm), and drawing off the supernatant.

Primers. The multiplex PCR utilizes primers specific for virulent YE based on the coding sequence of *ail* gene and the plasmid based *yadA* gene (13). The TaqMan PCR assay utilizes primers that amplified only the *ail* gene (unpublished).

Multiplex PCR. Each reaction (25 µl) contained DNA template (3 µl), reaction buffer, 200 nM dNTPs, 300 nM of each primer, and 1.25 U *Taq* polymerase. The reaction profile was as follows: 94°C for 3 min (initial denaturation); 94°C, 15 s (denaturation of template); 56°C, 15 s (primer annealing); 72°C, 15 s (primer extension) for 35 cycles; and 72°C for 3 min (final extension).

TaqMan PCR. Each reaction (50 µl) contained DNA template (5 µl), 3.5 mM $MgCl_2$, 2 mM dNTPs, 1× AmpliTaq Gold buffer and 1.25 U AmpliTaq Gold DNA polymerase. The primers and probe were added at 200 nM and 25 nM concentration, respectively. The reaction profile was as follows: 50°C for 2 min; 95°C for 10 min; 95°C, 15 s; 58°C, 1 min for 35 cycles.

PCR detection. Multiplex PCR products were detected by electrophoresing 5 µl of each reaction mix through a 1.5% agarose gel (100 V, 30 min) with Tris-Borate EDTA (1× TBE) as running buffer. The gels were stained with ethidium bromide, destained, and visualized in a Gel Doc 1000 system (Bio-Rad).

The TaqMan assay uses a fluorogenic probe. The amount of fluorescence detected is proportional to the amount of the amplified product (Figure 1). The system uses a computer algorithm to calculate ΔRn, the change in the fluorescent emission based on a background level fluorescence (12). A fluorescence threshold is determined and is equal to 10 standard deviations above the mean baseline fluorescence of the reactions containing no templates. Any reaction showing a ΔRn value above the threshold is considered positive at the end of 35 cycles. Once the amplification and analysis is complete, the 7700 ABI SDS (PE) system displays ΔRn values of all the samples in the 96-well format.

Results and Discussion

Hogs. The prevalence of YE in hog tissues collected before and after slaughter is shown in Table 1. Pathogenic YE was not detected by culture methods in any of the hog tissue samples tested. As summarized in Table 1, the multiplex PCR method detected YE in 2% of fecal samples and 1% of tonsil scrapings collected before slaughter. After slaughter, 3.7% of the tonsils were positive. Of the carcass swabs tested, 1.6% contained YE, which may reflect fecal contamination of the carcass during slaughter. The highest prevalence of YE was in ileocecal lymph nodes both by multiplex PCR (7%) and TaqMan assay (55%), compared with its presence in the other lymph nodes (ventral thoracic and superficial inguinal lymph nodes).

Processed pork products. YE was detected in chitterling samples (n=350) by bacteriological culture method (8%), by multiplex PCR (27%), and by TaqMan assay (79%) (Table 1). YE was not isolated in ground pork (n=350) by bacteriological culture method, but was detected with multiplex PCR (10%) and the TaqMan assay (44%).

Conclusion. YE was more frequently detected in ground pork and chitterlings than in freshly slaughtered hogs. This may suggest post-slaughter contamination of pork. The results suggest that the TaqMan Assay is several-fold more sensitive compared with the multiplex PCR assay and bacteriological culture method.

Acknowledgements

We extend our sincere thanks to Dr. Alissa. D. Jourdan for her guidance. We are indebted to Brad O. Chriswell and Karla Fenton for providing untiring and enthusiastic technical assistance in this study. We thank the Food Safety Consortium and National Pork Producers' Council for partial support.

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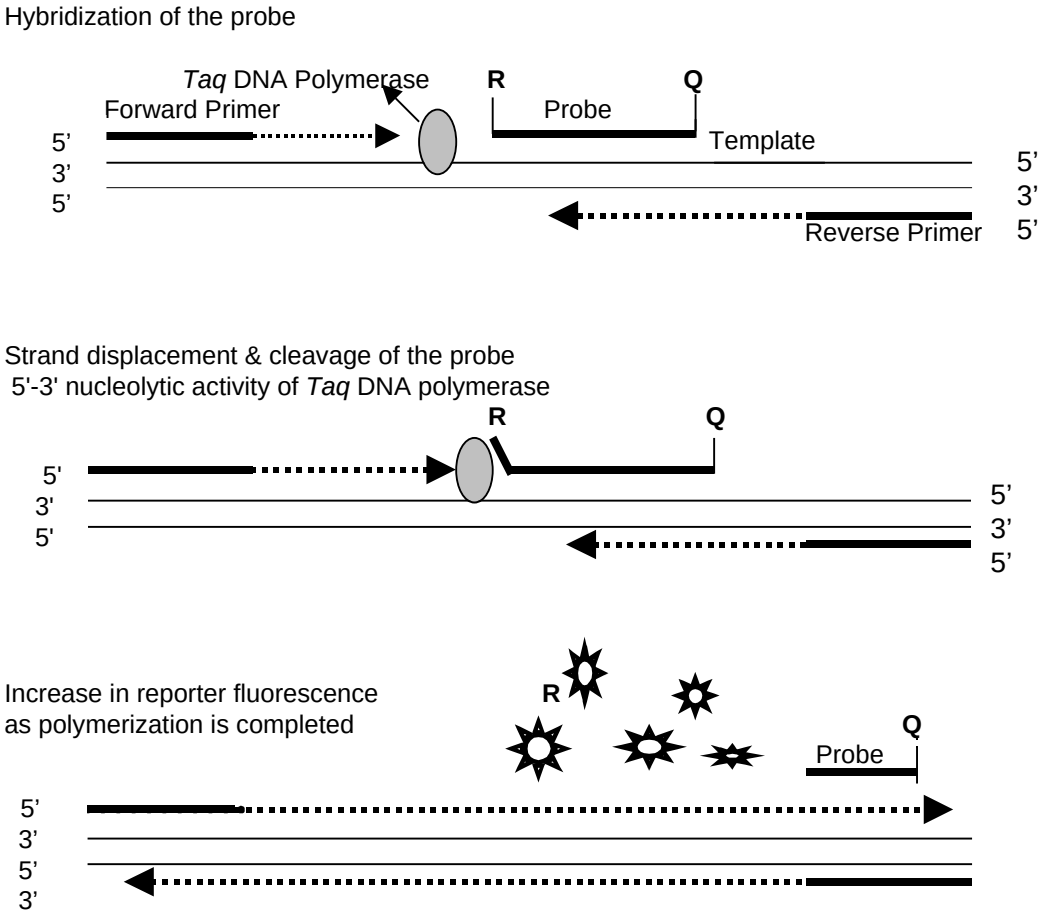

Figure 1. Principle of the TaqMan assay. [1] **R=reporter dye (FAM); Q=quencher dye (TAMRA)**

[1] Adapted from PE Applied Biosystem (12).

Table 1. Detection of *Yersinia enterocolitica* by culture, multiplex PCR and the TaqMan assays.

Specimen (n*~240) Hogs	Number of positive isolates (% of isolation)		
	Culture Method	Multiplex PCR	TaqMan PCR
Pre slaughter			
Fecal sample	0 (0%)	5 (2.0%)	100 (42%)
Tonsil scrapings	0 (0%)	3 (1.0%)	111 (46%)
Post slaughter			
Ileocecal LN	0 (0%)	17 (7.0%)	131 (55%)
Tonsils	0 (0%)	9 (3.7%)	125 (52%)
Rectal contents	0 (0%)	3 (1.0%)	114 (47%)
Cecal contents	0 (0%)	0 (0.0%)	102 (42%)
Carcass Swab	0 (0%)	4 (1.6%)	106 (44%)
Thoracic LN	0 (0%)	2 (0.8%)	94 (39%)
Superficial inguinal LN	0 (0%)	3 (1.3%)	98 (41%)
Processed pork products			
Chitterlings (n*=350)	27 (8.0%)	95 (27%)	278 (79%)
Ground pork (n*=350)	0 (0%)	35 (10%)	155 (44%)

n* is the number of samples of each specimen tested.