A PCR ELISA Method For The Detection Of Yersinia Enterocolitica

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Summary and Implications

The ELISA assay is a more rapid and sensitive method for PCR product detection than conventional gel electrophoresis. The PCR/ELISA allows for the processing of numerous samples, is relatively inexpensive and eliminates the need for electrophoretic and photographic equipment as well as the use of potential carcinogens such as ethidium bromide which is utilized in the gel electrophoretic detection of PCR products. The PCR ELISA is suitable for the rapid processing of samples required for field surveys of hogs and pork products.

Introduction

Yersinia enterocolitica is a zoonotic organism affecting both humans and livestock. In humans, a Y. enterocolitica infection can result in severe abdominal pain, fever, diarrhea, headache and vomiting, which mimic appendicitis. Because swine are a source of pathogenic Yersinia (De Giusti et al., 1995; Kapperud et al., 1991), we anticipate the need for rapid methods to determine the prevalence of Y. enterocolitica in large surveys of pork products or of market-weight hogs.

A PCR ELISA has been described for the field diagnosis of filarial infections (Nutman et al., 1994). The PCR ELISA is based on immobilization of biotin-labeled PCR products to streptavidin-coated wells of a microtiter plate. The PCR product is seen using a flouresceineated internal oligonucleotide probe specific to the amplicon, an enzyme-conjugated antibody, and a substrate-based colorimetric detection system (Nutman et al 1994).

The sequence of the ail gene, which is present only in virulent strains of Y. enterocolitica (Miller et al., 1989), has been previously reported (Feng et al., 1992).

Because the PCR ELISA has been utilized in field surveys, we investigated the feasibility of adapting this format to the detection of virulent Y. enterocolitica. Herein we describe a PCR ELISA, which targets the ail gene, in utilizing biotin-labelled PCR primers and a fluorescein-tagged internal probe.

Materials and Methods

Bacterial strains: The following strains were used to determine the specificity of the reaction: Yersinia enterocolitica ATCC 9610, 23175, 27729, 27739, 29913, 35669, 49397, and 55075; and Y. enterocolitica field strains NADC 5051, 5052, 50553, 50545, 5055, 5056, and 5057; Yersinia ruckeri NADC 3179 and 3182; Yersinia fredricksenii NADC 3183, Yersinia pseudotuberculosis NADC 3184; and Listeria monocytogenes NADC 3185.

Primer selection: Two PCR primers were selected which target the ail gene of Y. enterocolitica (Feng et al., 1992). The 5'-Biotin (BIOT) labeled primer (5'-BIOT-TTAATGTGTACGCTGCGAGTG-3') and the 3' primer (3'-CTGCGAAGTATACTTATGAGG-5') were commercially synthesized (Operon Technologies Alameda, CA) These primers amplify a 425-base pair (bp) product of the ail gene (Feng et al., 1992).

Preparation of bacterial DNA for PCR: Bacterial strains were frozen (-800(C) in glycerol. For bacterial growth, a loopful of bacteria was transferred directly from storage vials to cefsulodin-irgasan-novobiocin agar (CIN, Unipath) and incubated (300(C, overnight). A second loopful of bacteria was suspended in 200 ul. of 1 mM. disodium ethylene diamine tetraacetate-2H20 (EDTA), 10 mM. Tris, pH 8 (TE) and boiled (1000 C, 10 min.) in an aluminum block heater. The resultant cell suspensions were pelleted (13,600 x g. 2.5 min) and placed on ice.

PCR: A 5 ul. aliquot of the supernatant from the boiled cell suspension was used as the DNA template in a PCR. Each 50 ul. reaction mixture contained sterile dH20 (37.75 ul.), 10X reaction buffer (5 ul., Boehringer-Mannheim), nucleotide mix (1 ul., Boehringer-Mannheim), Taq polymerase (0.25 ul., Boehringer-Mannheim), the 5' biotin labelled and the 3' unlabelled PCR primer (0.5 ul. of each). The reaction mixture was overlaid with a drop of mineral oil and incubated in a DNA thermal cycler (Perkin-Elmer). The samples were initially denatured (940 C, 4 min.) and subjected to 35 cycles of amplification. Each amplification cycle consisted of denaturation (940 C, 1 min.), primer annealing (570 C, 2 min.), and primer elongation (720 C, 2 min.) as described (Feng 1992). After the final amplification cycle, the samples were heated (720 C, 5 min.) as a final primer elongation step. Following the DNA amplification, 5 ul. of 100 mM. EDTA was added to prevent DNA degradation (Uyeda unpublished data).

Electrophoresis analysis: PCR samples were analyzed by conventional agarose gel electrophoresis as follows. A 5 ul. aliquot of each PCR sample and 0.5 ul. of tracking dye was loaded on to a 1.5% agarose gel in Tris-borate-EDTA buffer (10 mmol-1 Tris, 2 mmol-1 EDTA, 90 mmol-1 Boric Acid, pH 8.5; TBE). DNA molecular weight marker VI (Boehringer Mannheim) was used as the base pair size reference. The gel was subjected to 80V. for 50 minutes in a horizontal bed (8.3 x 6.0 cm.) with TBE as the running buffer. The gel was stained with ethidium bromide (12 min.) and rinsed in dH20 (10 min.). The PCR products were visualized on a ultraviolet transilluminator light box (Fotodyne) and photographed using a red filter (Kodak 23A,) with Polaroid Film.

ELISA analysis: An ELISA was performed as described (Nutman et al. 1994). A fluorescein (FL) labeled oligonucleotide probe (5'- FLCTCCCCAGTT TCAT CGAGTTCFL-3') was designed for hybridization to a portion of the ail gene based on the previously published sequence (Miller et al., 1990).

Results and Discussion

The PCR-ELISA was adopted to detect virulent (ail -bearing) strains of Y. enterocolitica. The immobilization of the biotin-labelled PCR product to the streptavidin coated plate and the subsequent detection of that product by the fluourescein-labelled ail internal probe is depicted in Figure 1.

As summarized in Table 1, the PCR-ELISA was at least as specific as gel electrophoresis in detecting the 425 bp. product of the ail gene. PCR amplification occurred only when strains of Y. enterocolitica which harbored the ail gene were used as the template. Six of the seven ATCC reference strains yielded a PCR product. Failure to amplify strain ATCC 9610, which was acquired by ATCC in 1939, may be due to the loss of this virulence attribute upon multiple passages. Seven of the nine field strains also gave a positive reaction. No attempt had been made previously to determine the virulence of these field strains in mice. No amplification occurred with strains of Yersinia ruckeri, Y. pseudo-tuberculosis, Y. fredricksenii, Y. kristensenii, or L. monocytogenes. A comparison of the specificity of the PCR reaction by gel electrophoretic and ELISA detection is shown in Figure 2.

The sensitivity of the assay was evaluated using tenfold serial dilutions of Y. enterocolitica. As shown in Figure 3, the PCR ELISA was as sensitive as gel electrophoresis in detecting as few as 6 CFU per reaction mixture (50 ul.).

The ability of PCR ELISA to detect Y. enterocolitica experimentally inoculated into retail purchased ground pork was determined. Pork was seeded with Y. enterocolitica, homogenized and then plated on to CIN selective agar. After incubation (18 hr., 300 C), colonies were swiped from the most heavily populated quadrant of each CIN plate and prepared for PCR amplification. Gel electrophoresis showed the 425 bp. product in pork originally seeded with 4.9 x 104 CFU. The ELISA detected the ail gene in plates seeded with 4.9 x 103 CFU. The PCR ELISA was at least as sensitive as gel electrophoresis in detecting Y. enterocolitica among the indigenous bacterial flora of ground pork.

PCR ELISA provides a rapid method to survey large numbers of swine for the presence of pathogen (ail-bearing) strains of Y. enterocolitica. This method utilizes primers targeting the ail gene and an internal probe to confirm the identity of the PCR product. The specificity of the method is comparable to Southern blot hybridization (Nutmann et al., 1994). The colorimetric assay is comparable in sensitivity to gel detection and provides a quantitative estimate of titers of virulent Y. enterocolitica in samples.

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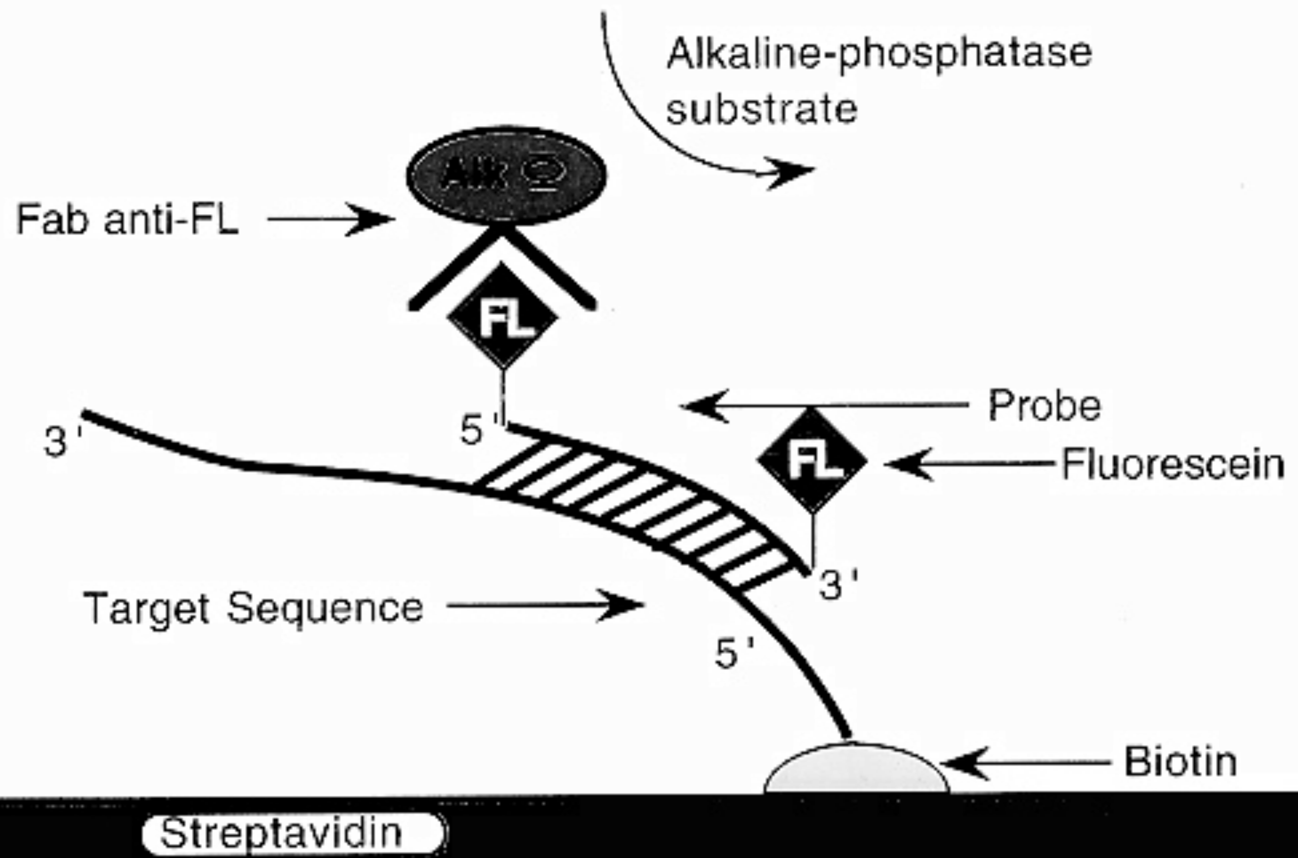
Table 1. Comparison of the specificity of the PCR-ELISA and gel electrophoresis for detection of the virulent ail-gene PCR product (425 bp.).

Species	ELISA	Gel Electrophoresis
Yersinia enterocolitica		
ATCC 9610	-	-
ATCC 23175	+	+
ATCC 27729	+	+
ATCC 27739	+	+
ATCC 29913	+	+
ATCC 49397	+	+
ATCC 55075	+	+
NADC 3181	+	+
NADC 3182	+	+
NADC 5051	+	+
NADC 5052	-	-
NADC 5053	+	+
NADC 5054	+	+
NADC 5055	+	+
NADC 5056	+	+
NADC 5057	-	-
Yersinia kristensenii ATCC 35669	-	-
Yersinia ruckeri		
NADC 3179	-	-
NADC 3180	-	-
Yersinia pseudotuberculosis NADC 3184	-	-
Y. fredricksenii NADC 3183	-	-
Listeria monocytogenes NADC 3185	-	-

Figure 1. Schematic of the enzyme-linked immunosorbent assay (ELISA)-based detection method for PCR products. Adapted from Nutman et al, 1994.

Figure 2. Specificity of gel electrophoresis and ELISA for detection of ail gene of virulent Yersinia enterocolitica.

Figure 3. Sensitivity of gel electrophoresis and ELISA for detection of ail gene of virulent Yersinia enterocolitica.


PCR-ELISA Detection System
Alkaline-phosphatase
substrate
Fab anti-FL
FL
Probe
FL
Fluorescein
3'
5'
3'
Target Sequence
5'
Biotin
Streptavidin

1.5% Agarose Gel Electrophoresis

CFU/Reaction

MW VI, 1.3×10^6, 1.3×10^5, 1.3×10^4, 1.3×10^3, 48.8, 5.75, 0.5, 0.05, 0, 0, Negative Control, Positive Control, MW VI

2176, 1766, 1230, 1033, 653, 517, 453, 394, 298

1.5% Agarose Gel of PCR products from dilutions of pure culture.

ELISA

+	+	+	+	+	+	-	-	-	-	Neg con	Pos con
1.3×10^6	1.3×10^5	1.3×10^4	1.3×10^3	48.8	5.75	0.5	0.05	0	0	Negative Control	Positive Control

CFU/Reaction

ELISA of PCR products from dilutions of pure culture.

1.5% Agarose Gel Electrophoresis

MW VI
Y. ruckeri
Y. ruckeri
Y. enterocolitica
Y. enterocolitica
Y. frederiksenii
Y. pseudotuberculosis
L. monocytogenes
Y. enterocolitica
Y. enterocolitica
Y. pseudotuberculosis
Neg. control
Pos. control
MW VI

2176
1766
1230
1033
653
298

1.5% Agarose Gel of PCR products from templates of *Yersinia* species and one *Listeria monocytogenes.*

ELISA

Y. ruckeri	Y. ruckeri	**Y. enterocolitica**	**Y. enterocolitica**	Y. frederiksenii	Y. pseudotuberculosis	L. monocytogenes	**Y. enterocolitica**	**Y. enterocolitica**	Empty	Neg. control	Y. pseudotuberculosis
-	-	+	+	-	-	-	+	+	-	No DNA	-

ELISA of PCR product from DNA template of *Yersinia* species and one *Listeria monocytogenes.*