Detection of *Yersinia enterocolitica* in Pigs and Pork Products

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Introduction

Yersinia enterocolitica is a zoonotic organism affecting both humans and livestock. In humans, *Y. enterocolitica* infection can result in severe abdominal pain, which mimics appendicitis. Swine have been implicated as the principal animal reservoir for human pathogenic *Y. enterocolitica*. Infections in pigs may localize in the tonsils (3). Several outbreaks of yersiniosis have involved consumption of pork (6, 9). Because swine are a source of pathogenic *Yersinia* (3, 5), rapid methods are needed to determine its prevalence in surveys of pork or of market weight hogs.

Three enrichments have been described in the literature. Irgasan, ticarcillin, and potassium chlorate (ITC) (11), modified trypticase soy broth (MTSB) (1), and Phosphate buffered saline (PBS) (12). However, the sensitivity of these methods has not been compared.

The sequence of the *ail* gene, which is present only in virulent strains of *Y. enterocolitica*, has been previously reported (7). The PCR primers amplifying the *ail* gene sequence have been described (4), and PCR detection of the *ail* gene has been used to confirm typical bulls-eye colonies grown on cefsulodin-irgasan-novobiocin (CIN) agar after enrichment (1).

A PCR ELISA has been described for the field diagnosis of filarial infections (8). The PCR ELISA is based on immobilization of PCR products to streptavidin-coated wells of a microtiter plate. The PCR product is visualized using a biotin-labeled internal oligonucleotide probe specific to the amplicon, an enzyme-conjugated antibody, and a substrate-based colorimetric detection system (8). We have adapted this format to the detection of *Y. enterocolitica* (10). The present study made use of digoxigenin labeled PCR products providing good sensitivity and a streamlined detection procedure.

The purpose of this study was to optimize the PCR ELISA assay that targets the amplification of the *ail* gene. Secondly, we wished to compare ITC, MTSB, and PBS enrichments for the detection of *Y. enterocolitica* in pigs and pork products (Figure 1).

Materials and Methods

Bacterial strains. The following *Y. enterocolitica* strains were obtained from the National Animal Disease Center Reference Collection. Serotypes are indicated in parenthesis: NADC 5559 (O:4,32), NADC 5560 (O:8), NADC 5561 (O:9), NADC 5562 (O:18), NADC 5563 (O:20), NADC 5564 (O:21), NADC 5565 (O:13), NADC 5566 (O:5,27), NADC 5567 (O:1,2,3), NADC 5569 (O:2,3), NADC 5570 (O:3) and NADC 5571 (O:3). Strain NADC 5571 was a human isolate obtained from a yersiniosis outbreak that involved the consumption of contaminated chitterlings. These strains were originally obtained from the Centers for Disease Control, Atlanta, GA. Additional *Yersinia* strains also were evaluated: *Y. aldovae*: NADC 5612, *Y. bercovieri*: NADC 5615, *Y. frederiksenii*: NADC 5616, *Y. intermedia*: NADC 5619, *Y. kristensenii*: NADC 5622, and *Y. mollaretii*: NADC 5625.

Enrichments. The protocols, including incubation times, for ITC, PBS, and MTSB are diagramed in Figure 1. The ITC enrichment broth is 10 g of tryptone, 1 g of yeast extract, 60 g of $MgCl_2 6H_2O$, 5 g of NaCl, 1 g of $KClO_3$, and 5 ml of 0.2% malachite green in 1 L of water (11). Enrichment was conducted at 25˚C for 48 hours. MTSB is 30 g of TSB (Difco), 2.5 g of yeast extract, and 2 g of sodium desoxycholate in 1 L of water, (pH 7.6). Following 24 hours of enrichment in MTSB at 15˚C, irgasan was added at a concentration of 4 µg/ml, and followed by 48 hours of additional incubation (1). PBS (pH 7.4) enrichments were incubated at 4˚C for 7 days (12).

At the completion of each enrichment a 1 ml aliqout of each was removed and DNA concentrated by a guanidine DNA extraction protocol. This step was included because earlier studies indicated the presence of substances in ITC which inhibited the PCR reaction. A 100 µl aliqout also was plated onto CIN agar (Oxoid). After incubation (overnight, 28˚C) CIN plates were examined for the characteristic bulls-eye colonies of *Yersinia*. Characteristic colonies were selected for PCR analysis (Figure 2).

Comparative sensitivity of enrichments. To test the sensitivity of each of the methods, 10-fold serial dilutions of *Y. enterocolitica* NADC 5571 (serotype O:3) were made in 10% ground pork (Figure 1). The suspensions were incubated (1 hour, 4˚C). One milliliter of each dilution (10^2 through 10^7) of this experimentally contaminated pork suspension was seeded into each of the three enrichments (10% vol/vol). Enrichments were as described above.

Hog tonsils. To compare the efficacy of enrichments, 107 hogs were sampled from three premises in Iowa: farms A (n=58 samples), B (n=30 samples), and C (n=19 samples). Tonsils were excised, homogenized, and seeded (10% wt/vol) into ITC, MTSB, and PBS enrichments. Enrichment samples were plated to CIN and a 1 ml aliqout treated with

guanidine extraction to remove PCR inhibitors.

Genomic DNA extraction. The guanidine extraction procedure was based on a DNA protocol using silica particles (2). Briefly, 1.5 ml of enrichment media was centrifuged (5 s, 13,000 x *g*) to pellet most of the large particular matter. One milliliter of the supernatant was harvested and recentrifuged (1 min, 13,000 x *g*) to pellet the bacteria. The supernatant was discarded and the pellet was resuspended in 1 ml of DNA binding solution (6 M guanidine hydrochloride, and 1% diatomaceous earth). The pellet was washed twice using 1 ml of wash solution (50% EtOH, 200 mM NaCl, 50 mM Tris-HCl pH 7.4, and 10 mM EDTA). Following the second wash, the pellet was centrifuged (30 s, 13,000 x *g*) and residual wash solution was aspirated. The pellet was then resuspended in 50 µl of water and heated (60˚C for 10 minutes). The diatomaceous earth was then pelleted (30 s, 13,000 x *g*) and the eluted DNA (5 µl) was used as a template for PCR. To process bacteria plated on CIN, a colony swipe was collected with a cotton swab, resuspended in 1 ml of PBS, and subjected to guanidine DNA isolation, as described above for enrichments

Primers. Primers specific for *Y. enterocolitica* were derived from the coding sequence of the *ail* gene (Feng, 1992). The primers were as follows: upper=5'-TTAATGTGTACGCT-GCGAGTG-3', lower=5'-GGAGTATTCATATGAAGC-GTC-3'. The combination of the two primers resulted in the amplification of 425-bp product.

Specificity. The specificity of the PCR was evaluated using a battery of *Yersinia* strains (Figure 3). *Yersinia* strains were streaked out on CIN agar and grown overnight at 28˚C. Following extraction of the DNA template, each sample was subjected to the standard PCR procedure as well as digoxigenin labeled for the PCR ELISA. Products from each PCR were then used for either gel detection or ELISA.

PCR. Five microliters of each DNA extraction was used as a template in a 50 µl reaction. Each reaction contained appropriate reaction buffer, 200 µM dNTPs, 2 µM of each primer, and 1.25 U *Taq* polymerase. The reaction profile was as follows: 94˚C; 3 min (94˚C; 15 s, 57˚C; 15 s, 72˚C; 25 s for 30 cycles) and 72˚C; 5 min.

Gel-based detection. Five microliters of each PCR reaction was electrophoresed through 2% agarose. The gels were stained with ethidium bromide, briefly destained, and visualized under ultraviolet light by using a Gel Doc 100 (Bio-Rad) system.

PCR ELISA detection. PCR ELISA reactions were performed using the PCR ELISA DIG-Detection System (Boehringer-Mannheim), according to the manufacturers protocol (Figure 4). As shown in Figure 3, PCR products were digoxigenin labeled using dig-dUTP during the amplification. Ten microliters of the PCR reaction was then used for the detection procedure that involved the hybridization of an internal biotinylated probe (5'-CTCC-CCAGTTATCATCGAGTTC-3') to *ail*-based PCR product generated by the primers described above. Hybridization was conducted in streptavidin-coated microtiter plates (MTP), allowing for the biotin-conjugated hybrids to bind to the surface of the plates. After removal of unbound DNA by washing, the *ail*-based PCR products were detected using a digoxigenin-specific antibody and a colorimetric reaction ABTS (2,2 '-Azino-di-[3-ethrybenzthiazoline sulfonate (6)]). The microtiter plates were analyzed on a THERMOmax microplate reader at 405 nm (Molecular Devices). All experimental OD values were normalized to negative control samples.

Results and Discussion

A 425-bp amplicon was detected on gels only with *Y. enterocolitica* and not with other species of *Yersinia*. Based on an OD_{405} > 0.100, the PCR ELISA was as sensitive as gel electrophoresis in detecting the *ail* gene of *Y. enterocolitica* (Figure 3).

As shown in Figure 2, based on the presence of a 425 bp amplicon *Y. enterocolitica* was detected prior to enrichment in pork samples experimentally seeded with 10^4 CFU/ml. A 425-bp amplicon was seen in all enrichments samples seeded with 10^6 and 10^5 CFU/ml. However, *Y. enterocolitica* was detected in samples inoculated with 10^4 and 10^3 and enriched in ITC and MTSB but not in PBS. Based on levels of detection ITC and MTSB were considered equivalent. However, that ITC enrichment requires 2 days whereas MTSB involved a 3-day procedure, including the addition of irgasan, suggests that ITC is the least labor intensive of the assays evaluated. It has been previously reported that a limited number of serotypes grow in ITC. However, in this study ITC supported the growth of pathogenic serogroups O:3; O:4,32; O:8; O:9; O:18; O:20; O:21; O:13; O:5,27; O:1,2,3; and O:2,3 (data not shown). In earlier studies with *Y. enterocolitica* NADC 5231, when seeded with an identical inoculum, ITC enrichment generated 100-fold more colonies than MTSB. Previous work in our laboratory showed that although a 425-bp amplicon was not detected from ITC seeded with hog tonsils, *Y. enterocolitica* colonies were visible when an aliqout from that same sample of ITC was plated to CIN. This indicated the presence of *Taq* polymerase inhibitors in ITC. Guanidine extraction removed these inhibitory compounds. Thus, a PCR assay could be used to screen enrichments with subsequent time-consuming plating to CIN and colony isolation reserved only for positive broths.

The PCR assay was evaluated for its ability to detect *Y. enterocolitica* in swine tonsils obtained from three premises in Iowa (Figure 5). *Yersinia enterocolitica* was not detected

in tonsilar homogenates farms A and C. However, *Y. enterocolitica* was cultured from 67% of samples from farm B on three sampling dates (=groups). As shown in Figure 5, *Y. enterocolitica* was present in samples of group 1 (60%), 2 (80%), and 3 (50%). Again, the ELISA PCR assay appeared to be as sensitive as the gel-based detection method.

Conclusions

ITC appears to be the most sensitive of the three enrichments evaluated. Artificially ground pork could be detected from ITC enrichment after guanidine extraction to remove PCR inhibitors present in the food matrix. The PCR ELISA and gel detection appear equally specific and sensitive in detecting *Y. enterocolitica*.

References

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Detection of *Yersinia* From Ground Pork

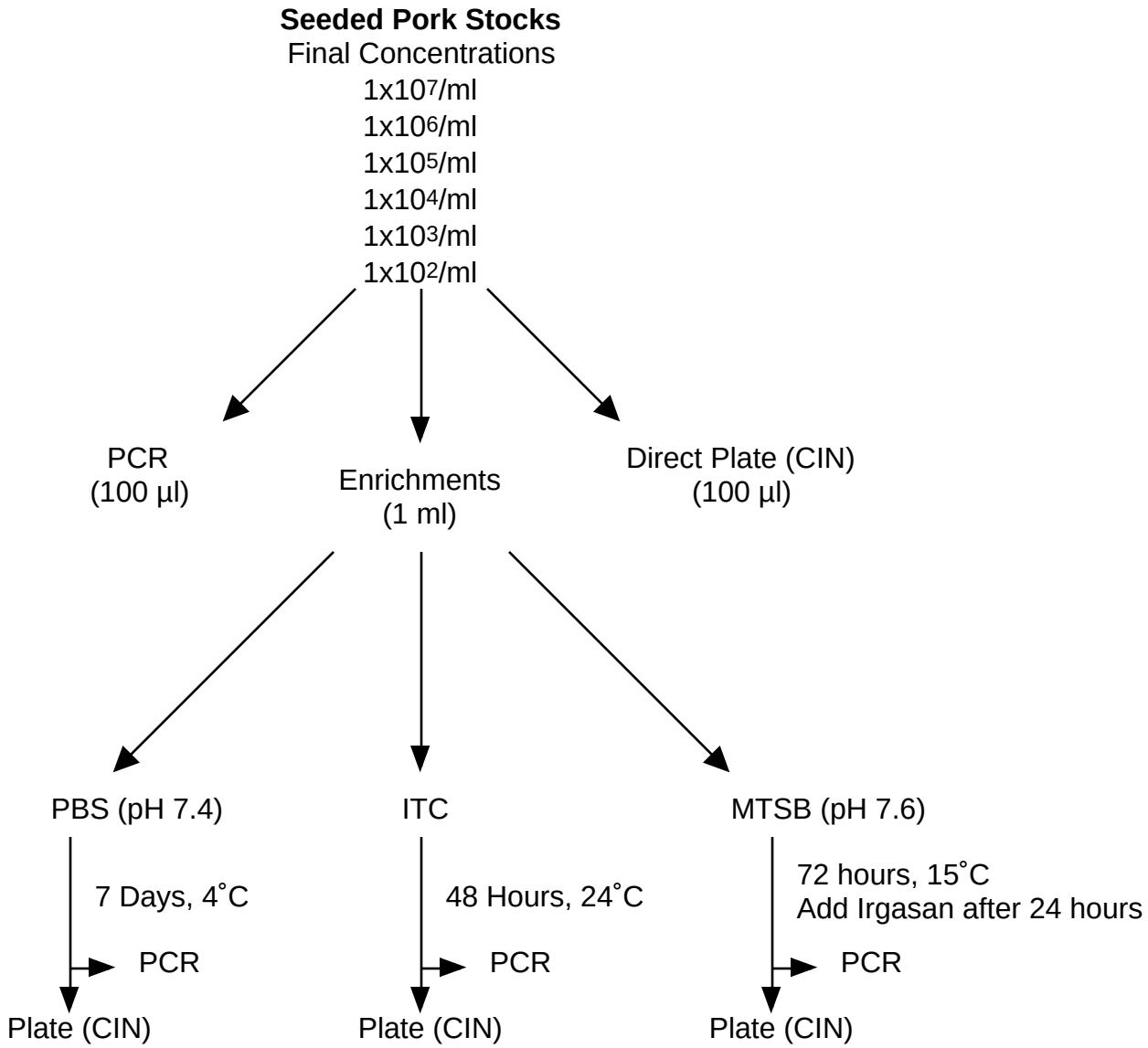
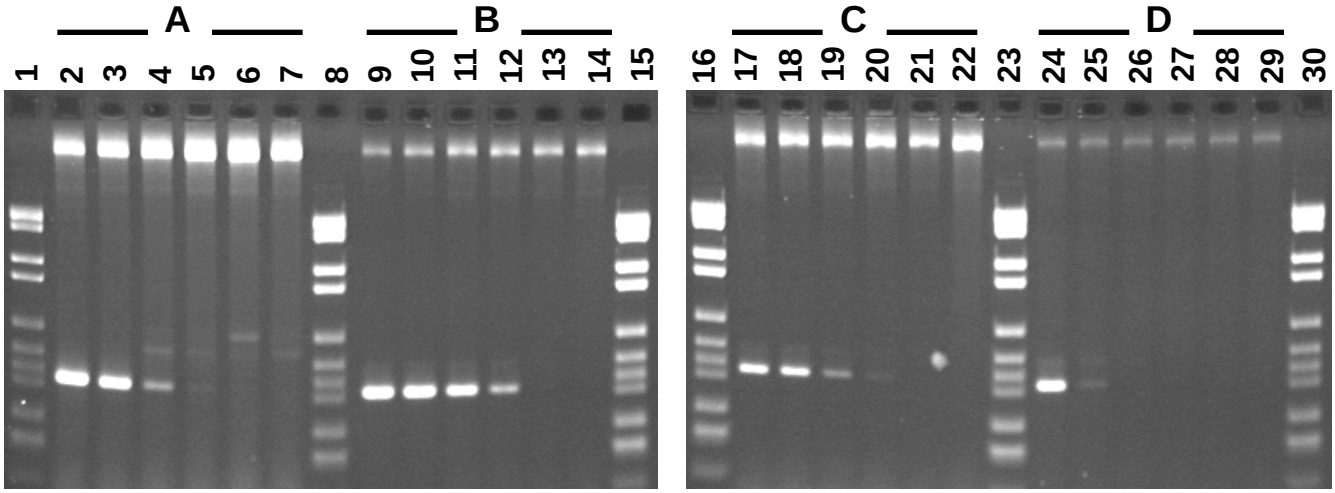


Figure 1 Outline of the procedure performed to test the efficacy of three enrichment methods to detect *Y. enterocolitica* in meat.

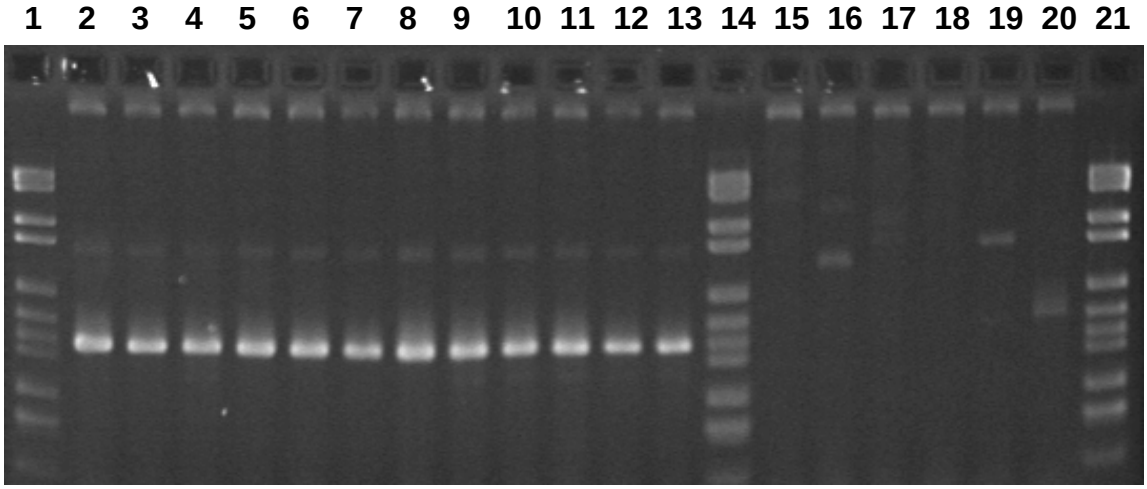
Yersinia Enrichments



Lane	Enrichment	Original Titer	Lane	Enrichment	Original Titer
1			16		
2	Direct Plate (A)	10^7	17	MTSB (C)	10^6
3	↓	10^6	18	↓	10^5
4		10^5	19		10^4
5		10^4	20		10^3
6		10^3	21		10^2
7		10^2	22		10^1
8			23		
9	ITC (B)	10^6	24	PBS (D)	10^6
10	↓	10^5	25	↓	10^5
11		10^4	26		10^4
12		10^3	27		10^3
13		10^2	28		10^2
14		10^1	29		10^1
15			30		

Figure 2 Detection of *Y. enterocolitica* dilutions in ground pork using direct plating (A), ITC (B), MTSB (C), and PBS (D) enrichments.

Screen of All *Yersinia*

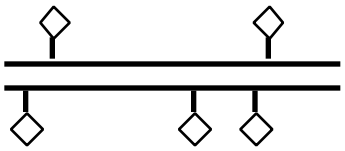


Lane	Strain	Serotype	ELISA OD
1			
2	*Y. enterocolitica*	O:4,32	1.093
3	*Y. enterocolitica*	O:8	0.786
4	*Y. enterocolitica*	O:9	0.833
5	*Y. enterocolitica*	O:18	0.951
6	*Y. enterocolitica*	O:20	0.852
7	*Y. enterocolitica*	O:21	0.478
8	*Y. enterocolitica*	O:13	0.668
9	*Y. enterocolitica*	O:5,27	0.602
10	*Y. enterocolitica*	O:1,2,3	0.518
11	*Y. enterocolitica*	O:2,3	0.534
12	*Y. enterocolitica*	O:3	0.508
13	*Y. enterocolitica*	O:3 H	0.469
14			
15	*Y. aldovae*		0.032
16	*Y. bercovieri*		0.034
17	*Y. frederiksenii*		0.032
18	*Y. intermedia*		0.026
19	*Y. kristensenii*		0.035
20	*Y. mollaretii*		0.028
21			

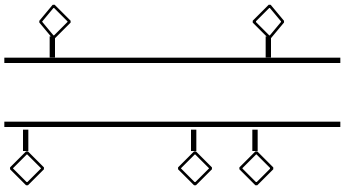
Figure 3 Detection of various *Yersinia* species using *ail*-specific primers. Detection was performed using gel-based analysis and PCR ELISA. Lanes 2-13 represent various serotypes of *Y. enterocolitica*. Lanes 15-20 are other non-*enterocolitica Yersinia* species.

Yersinia enterocolitica PCR ELISA

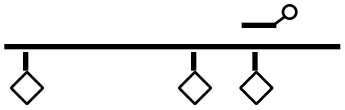
PCR amplification of *ail* fragment
Incorporation DIG-dUTP



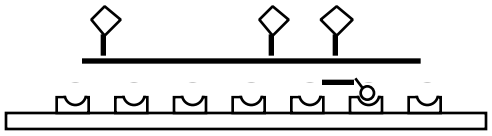
Denaturation of PCR product



Hybridization of biotinylated capture probe



Binding of hybrid to streptavidin coated MTP



Detection with Anti-DIG POD and ABTS substrate

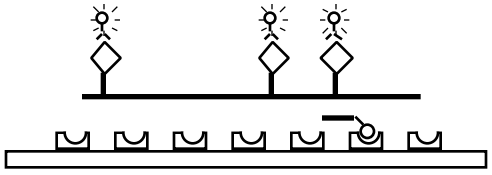
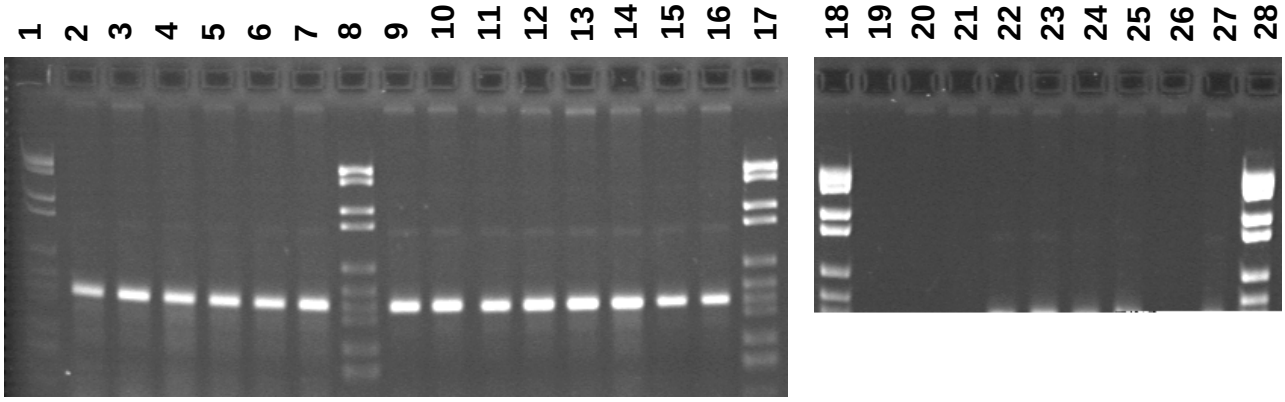


Figure 4 Schematic outline of the PCR ELISA procedure used to detect *Y. enterocolitica*. DIG-dUTP, Digoxigenin labeled dUTP; MTP, Microtiter plate; Anti-DIG POD, Peroxidase conjugated antibody against digoxigenin; ABTS, 2,2 '-Azino-di-[3-ethrybenzthiazoline sulfonate (6)].

Sample Farm B



Lane	Group	Pig	ELISA OD
1			
2	1	1	3.203
3		2	3.051
4		3	3.222
5		5	3.091
6		6	2.610
7		7	2.722
8			
9	2	1	1.390
10		2	1.232
11		3	1.608
12		4	1.898
13		7	2.449
14		8	2.481
15		9	1.482
16		10	1.345
17			
18			
19	3	1	0.036
20		2	0.041
21		3	0.074
22		4	1.439
23		6	2.618
24		7	2.270
25		8	1.882
26		9	0.027
27		10	0.894
28			

Figure 5 Sample farm with high incidence of pigs infected with *Y. enterocolitica* as determined by gel-based and PCR ELISA detection following enrichment in ITC.