Detection of *Yersinia* Species in Pig Tonsils by a 5´ Nuclease Fluorogenic (TaqMan) Polymerase Chain Reaction Assay Specific for the 16S rRNA Gene

Alissa Jourdan, Margarita Davila Marichal, Laura Byl, and Irene Wesley
National Animal Disease Center, Ames, IA

ASL-R1791

Summary and Implications

We developed and optimized a fluorogenic 5′ nuclease polymerase chain reaction assay, which is specific for the 16S rRNA present in the majority of *Yersinia* species. The optimized assay uses the reporter dye VIC and the quencher dye TAMRA. The assay amplified all species of *Yersinia*, except *Y. ruckeri*, which is fish pathogen. The assay detects five picograms of *Yersinia* DNA. The assay was used to screen tonsil swabs (n = 533) collected during the National Animal Health Monitoring System (NAHMS) Swine 2000 study. Tonsil swabs were received from 101 farms from 17 states and shipped to National Animal Disease Center for processing. Of the 553 tonsil samples screened, 61.8% (342) were positive for the 16S rRNA gene of *Yersinia*.

Introduction

The genus *Yersinia* encompasses 10 species: *Y. aldovae, Y. bercovieri, Y. enterocolitica, Y. frederiksenii*:, *Y. intermedia, Y. kristensenii, Y. molaretti, Y. pestis, Y. pseudotuberculosis,* and *Y. rukeri*. The species *Y. pestis, Y. enterocolitica,* and *Y. pseudotuberculosis* cause zoonotic infections; *Y. intermedia, Y. kristensenii* and *Y. frederiksenii* are opportunistic human pathogens.

Y. pseudotuberculosis and *Y. enterocolitica* have been isolated from a variety of species, including pigs.

Y. enterocolitica is a human enteric pathogen that may be acquired by ingestion of contaminated food, water, or milk. *Y. enterocolitica* has been detected in ponds, lakes, and foods, such as pork, beef, tofu, ice cream, and milk. Most isolates are non-pathogenic. Swine are an important reservoir for serotypes O:3 and O:9 which are pathogenic for humans (1, 2, 6, 7). *Y. enterocolitca* colonizes the pig's oral cavity, tongue, and gut. Spillage of gut contents during processing may result in *Yersinia*-contaminated carcasses (4).

We developed a 5´ nuclease polymerase chain reaction (TaqMan) assay, which targets the 16S rRNA gene conserved in the majority of *Yersinia* species. The TaqMan assay uses an oligonucleotide probe, which is labeled at the 5´ end with a fluorogenic reporter dye and quencher dye at the 3´ terminus. The quencher dye suppresses fluorescent emission of the reporter dye on the intact probe (3,5). During PCR amplification, the probe anneals to the target sequence located between the forward and the reverse primer binding sites (6). During primer extension, the *taq* polymerase cleaves the probe. This cleavage liberates the fluorescent reporter from the quencher dye, which increases fluorescence.

The goals of this study were (1) to develop and optimize a 5´ nuclease assay for all *Yersinia* species, and (2) to use this assay to screen tonsil swabs collected from market weight hogs during the National Animal Health Monitoring System 2000 Swine study.

Materials and Methods

Primer and Probes. Primer and probe sequences that were used in this PCR assay encode for the 16S rRNA gene, which is present in all *Yersinia* species (Table 1). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). The probe (24-mer) was labeled at the 5′ end with fluorescent reporter dye VIC and at the 3′ end with the quencher dye TAMRA-6-carboxy-tetramethyl-rhodamine (PE Applied Biosystems, Foster City, CA).

Fluorogenic 5´Nuclease PCR Conditions. The PCR mixture contained 3 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.4 mM, 2.5 U of AmpliTaq Gold DNA polymerase (PE Applied Biosystems), and 1 X GeneAmp PCR Gold Buffer (150mM Tris-HCl, 500 mM KCl; PE Applied Biosystems). Five microliters of DNA template was added to make a total volume of 50 µl. The thermal cycling conditions were as follows: initial denaturation (95°C for 10 minutes) and 33 cycles of consisting of denaturation (95°C for 15 seconds) and primer annealing and extension (60°C for one minute) followed by an indefinite hold (25°C).

Analysis was completed on the ABI 7700 Sequence Detection System. Cycle 30 was set as an arbitrary cut off point. Any sample whose fluorescence exceeded the threshold before cycle 30 and showed logarithmic amplification was scored as positive. Any sample that did not cross the threshold by cycle 30 or did not display logarithmic amplification was scored as negative (Figure 1).

Determination of Specificity and Sensitivity. To determine specificity, DNA was prepared from representative species of *Yersinia* and other bacteria, as described previously (3). Sensitivity was based on PCR amplification of 10-fold serial dilutions of DNA prepared from *Y. enterocolitica*, as described (3).

*Sample Enrichment***.** Pig tonsil samples were collected during the NAHMS 2000 study. Tonsil swabs were collected by APHIS field veterinarians and placed into transport media and shipped on wet ice to NADC. Swabs (Copan) were placed in 10 ml of ITC *Yersinia* isolation media (2 days at room temperature) and an aliquot (100 µl) subcultured onto a CIN plate (2 days at room temperature).

*DNA Extraction***.** DNA was extracted from ITC media as follows. One milliliter was placed in a microfuge tube, centrifuged (2 minutes at 10,000 x g), and the supernatant was carefully removed. The pellet was resuspended in 200 µl of PrepMan reagent (PE Applied Biosystems) and placed in a heat block (102°C, 10 minutes). The tubes were centrifuged (two minutes at 10,000 x g) to pellet cell debris and the resultant supernatant was transferred into a new microcentrifuge tube and stored (-20°C). DNA extractions were prepared from all the ITC samples.

Results and Discussion

Optimization of TaqMan Assay. Initially, the probe was labeled at the 5' end with the reporter dye (JOE), which yielded inconsistent results. Substitution with the dye (VIC) significantly decreased background fluorescence. The fluorescently labeled probe (24-mer) hybridized with the 89-bp PCR product.

*Specificity of TaqMan Assay***.** The assay amplified 8 of the 10 species of *Yersinia*, including *Y. bercovieri*, *Y. enterocolitica*, *Y. kristensenii*, *Y. aldovae*, *Y. frederiksenii*, *Y. intermedia*, *Y. molaretti*, *Y. pseudotuberculosis*. It did not amplify *Y. ruckeri* or other non-*Yersinia* species by cycle 35 (Tables 2 and 3). *Y. pestis* was not tested. However, *Rahnella aquatilis*, which shares extensive homology with *Yersinia*, but is regarded as a separate genus, was amplified.

Sensitivity of TaqMan Assay. The assay amplified 5 pg of *Y. enterocolitica* DNA.

*Number of Positive Samples***.** The number of samples positive for *Yersinia* was based on screening ITC enrichment broth cultures. Of the 553 ITC samples analyzed, 62% (342) were found to be positive for the 16S rRNA gene conserved in the majority of *Yersinia* species.

The objective of this project was to develop and optimize a 5´ nuclease fluorogenic PCR assay targeting the 16S rRNA gene, which is conserved in the majority of *Yersinia* species. Although initially using JOE as the reporter dye, it was found that background fluorescence interfered with the analysis. The reporter dye VIC provided consistent results.

The optimized assay amplified eight of nine species of *Yersinia*, including *Y. enterocolitica* and *Y. pseudotuberculosis*, which have been reported in hogs. It did not amplify *Y. ruckeri*, which is a fish pathogen. However, *Rahnella aquatilis*, which shares extensive homology with *Yersinia*, was amplified. The assay detected 5 pg of purified *Y. enterocolitica* DNA.

The optimized assay was used to screen pig tonsils for *Yersinia* species. Of 553 samples tested, 62% (342) were positive for some species of *Yersinia*. This study indicates that species of *Yersinia*, including *Y. enterocolitica* and *Y. pseudotuberculosis*, may be present in tonsils of healthy market weight hogs in the US.

Acknowledgements

This study was funded in part by the National Pork Produces Council, the ISU Food Safety Consortium and the ISU Agriculture Minority Summer Internship Program.

References

1. Cover, T.L. and R.C. Aber. 1989. *Yersinia enterocolitica.* N Engl. J. Med. 321: 16 - 24.
2. Doyle, M.P., M.B. Hugdahl. and S.L Taylor. 1981. Isolation of virulent *Yersinia enterocolitica* from porcine tongues. Appl. Environ. Microbiol. 42: 661 - 666.
3. Jourdan, A.D., S.C. Johnson, and I.V. Wesley. 2000. Development of a fluorogenic 5' nuclease PCR assay for detection of the *ail* gene of pathogenic *Yersinia enterocolitica* . Appl. Enviro. Microbiol. 66: 3750 - 3755.
4. Kapperud, G. 1994 *Yersinia enterocolitica* infection. Handbook of Zoonoses, 2nd Ed. 343 - 353.
5. Livak, K., J. Maramro., and S. Flood. 1995. Guidelines for designing TaqMan fluorogenic probes for 5´ nuclease assays. ABI PRISM Sequence Detection Systems Users Bulletin.
6. Thibodeau, V., E.H. Frost., S. Chenier. and S. Quessy. 1999. Presence of *Yersinia enterocolitica* in tissues of orally-inoculated pigs and the tonsils and feces of pigs at slaughter. Can. J. Vet. Res. 63: 96 - 100.
7. Vishnubhatla, A., D.Y.C. Fung., R.D. Oberest., M.P. Hays., T.G. Nagaraja. and S.J.A. Flood. 2000. Rapid 5´ nuclease (TaqMan) assay for detection of virulent strains of *Yersinia enterocolitica*. Appl. Environ. Microbiol. 66: 4131 - 4135.

Table 1. List of primers and probe used in the assay

Primer/probe	Annealing site	Sequence (5′ ➪ 3′)
16S-F	557-572	GCA CGC AGG CGG TTT G
16S-R	620-646	CTC TAC AAG ACT CTA GCT TGC CAG TTT
16S-P	594-618	CGC GCT TAA CGT GGG AAC TGC ATT T

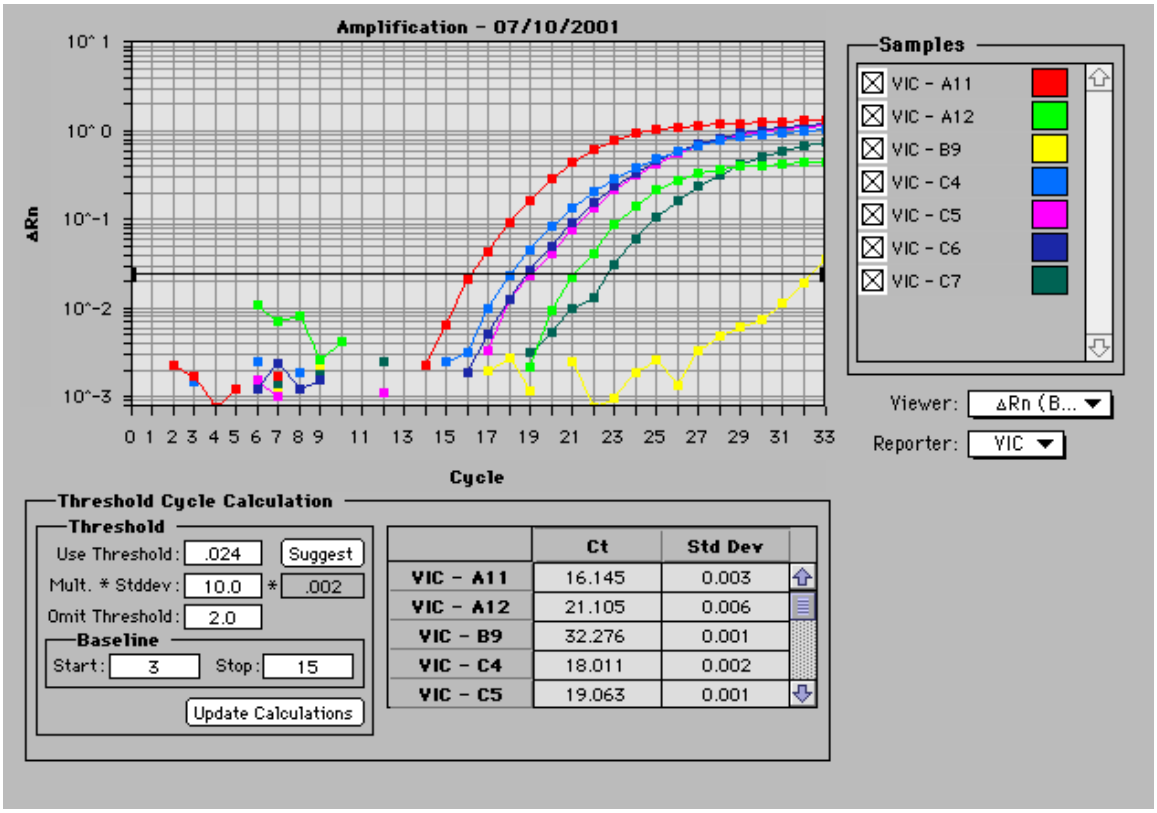

Figure 1. Analysis of NAHMS tonsil samples for the presence of the *ail*-bearing *Y. enterocolitica* by using a 5´ nuclease fluorogenic PCR (TaqMan) assay.

Sample Type	Sample	Conclusion
Known Positive	A11 & A12	Positive
Field Sample '+'	C4, C5, C6, & C7	Positive
Field Sample '-'	B9	Negative

TABLE 2. Summary of reactivity of strains used to determine specificity of *Yersinia* TaqMan assays. Strains were scored as positive after 35 amplification cycles.

Organism	Strain # NADC	16s
Y. bercovieri	5230	+
Y. enterocolitica	5231	+
Y. enterocolitica	5232	+
Y. enterocolitica	5233	+
Y. enterocolitica	5234	+
Y. enterocolitica	5235	+
Y. kristensenii	5236	+
Y. enterocolitica	5237	+
Y. enterocolitica	5559	+
Y. enterocolitica	5560	+
Y. enterocolitica	5561	+
Y. enterocolitica	5562	+
Y. enterocolitica	5563	+
Y. enterocolitica	5564	+
Y. enterocolitica	5565	+
Y. enterocolitica	5566	+
Y. enterocolitica	5567	+
Y. enterocolitica	5568	+
Y. enterocolitica	5569	+
Y. enterocolitica	5570	+
Y. enterocolitica	5571	+
Y. enterocolitica	5610	+
Y. enterocolitica	5611	+
Y. aldovae	5612	+
Y. aldovae	5613	+
Y. aldovae	5614	+
Y. bercovieri	5615	+
Y. frederiksenii	5616	-
Y. frederiksenii	5617	+
Y. frederiksenii	5618	+
Y. intermedia	5619	+
Y. intermedia	5620	+
Y. intermedia	5621	+
Y. kristensenii	5622	+
Y. kristensenii	5623	+
Y. kristensenii	5624	+
Y. molarettii	5625	+
Y. pseudotuberculosis	8119	+
Y. pseudotuberculosis	8120	+
Y. pseudotuberculosis	8121	+

Y. ruckeri	8122	-
Y. rucker	8123	-
Y. enterocolitica	8177	+
Y. enterocolitica	8178	+
Y enterocolitica	8179	+
Y. enterocolitica	8180	+
Y. enterocolitica	8181	+

TABLE 3. Summary of non-*Yersinia* strains used to determine specificity of TaqMan assay.

Organism	Strain# NADC	16s
E. coli	8176	-
E. coli	8252	-
Hafnia alvei	8526	-/+
Hafnia alvei	8527	-/+
Hafnia alvei	8528	-/+
Hafnia alvei	8529	-/+
S. aureus	3649	-/+
S. aureus	3690	-/+
L. monocytogenes	2045	-
L. monocytogenes	2052	-
P. vulgaris		-
K. pneumoniae		-
P. aeruginosa		-
E. cloacea		-
C. jejuni	5095	-
R. aquatilis	8571	+
R. aquatilis	8572	+

- No reaction at 35 cycles

-/+ Very weak reaction at 35 cycles. No reaction at 33 cycles

+ Strong reaction at 35 cycles (Expected reaction with Rahnella)