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# Enhanced Detection of Intracellular Organism of Swine Proliferative Enteritis, Ileal Symbiont Intracellularis, in Feces by Polymerase Chain Reaction

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**A sensitive assay based on amplification of a 319-bp DNA fragment of the intracellular bacterium of swine proliferative enteritis was developed for the detection of the organism in the feces of swine. A vernacular name, ileal symbiont intracellularis (IS-intracellularis), has recently been published for the intracellular bacterium, which was formerly known as a *Campylobacter*-like organism (C. J. Gebhart, S. M. Barnes, S. McOrist, G. F. Lin, and G. H. K. Larson, Int. J. Syst. Bacteriol. 43:533–538, 1993). As few as $10^1$ IS-intracellularis organisms purified from intestinal mucosa, or $10^3$ IS-intracellularis per g of feces, were detected. No amplification product was produced from a polymerase chain reaction performed on DNA extracted from the feces of healthy pigs. A 319-bp DNA fragment specific for IS-intracellularis was produced on amplification of DNA from the feces of pigs with experimental and naturally occurring proliferative enteritis.**

Proliferative enteritis (PE) is a diarrheal disease of growing swine (6 to 20 weeks of age) which occurs throughout the world. It can cause a decrease in the rate of weight gain, weight loss, and occasionally, death (16). The prevalence and economic impact of PE have not been determined, largely because of the lack of an accepted antemortem diagnostic technique (4, 7, 18, 23). Examination of the intestines of animals at slaughter has been of limited value, because PE usually occurs before slaughter (4, 18, 23), and gross examination and palpation of intestinal tracts have overestimated the occurrence of the disease (3).

The causative agent is not known. A curved, rod-shaped bacterium is always found within the enterocytes of affected pigs, but it has not been cultivated (12, 16). However, an antigenically and morphologically similar bacterium is found within the enterocytes of hamsters (*Mesocricetus auratus*) with PE (11) and has been cultivated in tissue culture. Pure cultures of that organism have transmitted PE in hamsters (20). The intracellular bacterium of swine, formerly known as a *Campylobacter*-like organism, has been given the vernacular name ileal symbiont intracellularis (IS-intracellularis) (5).

DNA sequences specific for IS-intracellularis were cloned and characterized (6). Nonradioactive probes prepared from these sequences detected IS-intracellularis in DNA extracted from swine feces with a sensitivity of $10^7$ IS-intracellularis per g (9). Amplification of DNA by the polymerase chain reaction (PCR) is exquisitely sensitive in the diagnosis of diseases caused by fastidious agents or agents whose identification is difficult or laborious, including *Treponema pallidum*, *Clostridium difficile*, *Mycobacterium leprae*, and *Mycobacterium paratuberculosis* (2, 8, 14, 22). Here, we describe a simplified extraction method for the recovery of DNA from bacteria in feces and a PCR-based assay for the detection of IS-intracellularis in feces. The use of this assay will facilitate disease monitoring in the field and experimental studies of the pathogenesis and epidemiology of PE.

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## MATERIALS AND METHODS

**Primers.** A 375-bp segment of a DNA fragment from the IS-intracellularis-specific DNA clone p78 (6) was sequenced (17) by using Sequenase and a commercial kit (Sequenase kit; U.S. Biochemical Corp., Cleveland, Ohio). Four primers of 20 nucleotides in length and flanking 279- and 220-bp sequences were synthesized on a DNA synthesizer (model 391; Applied Biosystems, Foster City, Calif.), as follows: primer A, 5′-TATGGCTGTCAAACACTCCG-3′; primer B, 5′-TGAAGGTATTGGTATTCTCC-3′; primer C, 5′-TTAC AGGTGAAGTTATTGGG-3′; and D, 5′-CTTTCTCATGTC CCATAAGC-3′. Primers A and B correspond to nucleotides 5 to 24 and 304 to 323, respectively, in the sequence of the cloned fragment of IS-intracellularis DNA. Primers C and D correspond to nucleotides 45 to 64 and 285 to 304, respectively, in the IS-intracellularis DNA fragment. The 375-bp sequence is deposited in GenBank (accession number L08049).

**PCR.** Reagents for the PCR were supplied in a commercial kit (Gene Amp; Perkin-Elmer Cetus, Norwalk, Conn.). Optimized reaction conditions consisted of 2 mM $MgCl_2$, 5% dimethyl sulfoxide, 30 ng of each of the external primers (A and B), and 1 U of *Taq* DNA polymerase in a 25-μl reaction volume. The concentrations of other reagents were as specified by the manufacturer (200 mM [each] deoxynucleoside triphosphates, 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.001% gelatin).

Sample DNA was dissolved in PCR buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10 mM NaCl). Ten-microliter sample aliquots were mixed with the reagents, heated to 100°C for 10 min, and cooled to 55°C, at which time 1 U of *Taq* polymerase was added. Reactions were continued for 35 cycles in a DNA thermal cycler (Perkin-Elmer Cetus), with each cycle consisting of 93°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Negative control samples without DNA were subjected to PCR amplification in all experiments.

Nested PCR was performed on 1 μl of each amplification product by using internal primers C and D and the same reaction conditions, step times and temperatures, and number of cycles as in the original amplification.

**Detection of PCR products.** Reaction products (3 μl) were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. An *Hae*III digest of φX174 replicative-form DNA (GIBCO Bethesda Research Laboratories, Gaithersburg, Md.) was used as a molecular marker.

For greater sensitivity and specificity, DNA was transferred to nylon membranes (Hybond N; Amersham Corp., Arlington Heights, Ill.) by Southern blotting (19), hybridized to a digoxigenin-labeled IS-intracellularis-specific DNA probe (p78), and detected by chemiluminescence by using commercial kits (Genius DNA Labeling and Detection Kit Nonradioactive and Lumi-Phos 530; Boehringer-Mannheim, Indianapolis, Ind.) as described previously (9). Alternatively, reaction products were reamplified with primers C and D and the reaction products were analyzed by electrophoresis, as described above.

**Diagnosis of PE.** The diagnosis of PE in all pigs was based on the histological examination of hematoxylin and eosin (H&E)- and silver-stained sections of ileum, cecum, and colon for the presence of characteristic proliferative lesions and IS-intracellularis (12, 13, 21). At necropsy, 3-cm samples of ileum ($n$ = 2) 30 cm apart were taken proximal to the ileocecal junction and were fixed in buffered 10% formalin. One sample each from the cecum and colon was taken and fixed in a like manner. Sections (5 μm) were cut from each tissue sample and stained with either H&E or Warthin-Starry (silver) stains. The H&E-stained sections were examined for loss of intestinal villi, the presence of inflammatory debris within the lumens of intestinal crypts, proliferation of enterocytes lining the crypts, and a decrease in the number of goblet cells. Silver-stained sections were examined for the presence of darkly staining, slender, curved, rod-shaped bacteria within the apical cytoplasm of enterocytes.

**Preparation and quantification of IS-intracellularis.** A suspension of semipurified IS-intracellularis was prepared, and IS-intracellularis was quantified as described previously (9). Briefly, the ileal mucosa from pigs with PE was scraped from the ileum and homogenized by 10 strokes in a Dounce tissue grinder (Dura-Grind Dounce tissue grinder; Wheaton Scientific, Millville, N.J.). The homogenate was centrifuged at 750 × $g$ for 10 min, and the supernatant was filtered sequentially through 6.6-μm, 1.2-μm (Uniflo; Schleicher & Schuell, Keene, N.H.), and 0.8-μm (Milex-PF; Millipore Products Division, Bedford, Mass.) filters. The filtrate was centrifuged at 8,000 × $g$ for 10 min, and the pellet was resuspended in phosphate-buffered saline (PBS). The final resuspended pellet was referred to as the infected mucosal filtrate.

Ten microliters of the infected mucosal filtrate was applied to a glass slide, spread over 1 $cm^2$, and air dried. The concentration of IS-intracellularis was determined by reaction with a specific monoclonal antibody (provided by G. H. K. Lawson and S. McOrist, Royal [Dick] School of Veterinary Studies, University of Edinburgh) and fluorescein isothiocyanate-labeled anti-mouse immunoglobulin G (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Md.) by published procedures (10).

**Preparation of mock-infected (spiked) feces.** IS-intracellularis-negative feces were obtained at slaughter from noninfected pigs, as determined by postmortem histological examination of ileal mucosa, or from an isolated high-health-status herd at the University of Minnesota, St. Paul. Fecal samples were collected from the rectum and large bowel at the postmortem examination. From the farm, fresh feces were collected from the concrete platform or by digitally stimulating the pig's rectum. The feces were divided into 5-g samples and were frozen at −80°C until use. Mock-infected (spiked) fecal samples were each prepared by adding 1-ml dilutions of the infected mucosal filtrate in PBS to 1 g of negative fecal samples to produce fecal samples containing $10^1$ to $10^6$ IS-intracellularis per g of feces, as determined by monoclonal antibody–indirect fluorescent-antibody counts of the infected mucosal filtrate. The feces and mucosal filtrate were vortexed for 1 min to ensure an even distribution of the IS-intracellularis. In the same manner, a negative control fecal sample was prepared by adding PBS to a negative fecal sample.

**Extraction of DNA from infected mucosal filtrate.** DNA was extracted by modifications of a procedure (guanidine thiocyanate [GuSCN]-diatomaceous earth [DE]) based on the binding of DNA to silicates in the presence of high concentrations of GuSCN (1). A 20% (wt/vol) DE suspension (50 μl) in 0.17 M HCl was vortexed with infected mucosal filtrate (50 μl) in a sterile microcentrifuge tube containing 950 μl of lysis buffer consisting of 5 M GuSCN, 22 mM EDTA, 0.05 M Tris HCl (pH 6.4), and 0.65% Triton X-100. The sample was held at room temperature for 10 min, vortexed, and centrifuged at 14,000 × $g$ for 20 s. The lysis buffer was drawn off with a pipette, and the pellet was washed twice in 5.5 M GuSCN and 0.05 M Tris HCl (pH 6.4). The pellet was washed twice in cold 70% ethyl alcohol and once in acetone. With each wash the pellet was vortexed until it was thoroughly dispersed. The acetone pellet was dried at 56°C for 15 min. The DNA from each tube was dissolved in 75 μl of PCR buffer, drawn off with a pipette, and stored at 4°C.

**Extraction of DNA from feces.** A fecal sample (~0.2 g) was collected with a sterile cotton swab, suspended in 1 ml of lysis buffer in a sterile microcentrifuge tube, and vortexed for 30 s to disperse the particles evenly. The sample was allowed to stand at room temperature for 1 h and was then centrifuged at 14,000 × $g$ for 20 s. The supernatant was drawn off and placed in a tube containing 50 μl of the DE suspension. Further processing was as described above for the extraction of DNA from mucosal filtrate.

**Experimental reproduction of PE in pigs.** Mixed-breed, 10-week-old pigs ($n$ = 4) were housed in the isolation facilities of the College of Veterinary Medicine. Pigs in one room ($n$ = 3) were orally inoculated with 40 ml of homogenized ileal mucosa from a pig with PE. An uninoculated pig was housed separately as an unexposed control. At 2, 3, and 5 weeks after inoculation, pigs were euthanized and necropsied. Tissue samples were collected and examined as described above in the section on the diagnosis of PE.

**Naturally occurring PE.** Tissue and fecal samples were collected from pigs with PE at necropsy and from a pig with diarrhea and suspected of having PE. Tissue and fecal samples were collected from normal appearing intestinal tracts of pigs at a slaughter plant (15). Tissue samples were fixed and examined as described above in the section on the diagnosis of PE. Fecal samples were placed on ice until they were returned to the laboratory and were stored at −20°C.

## RESULTS

Amplification of DNA extracted from 2 × $10^4$ IS-intracellularis from infected mucosal filtrates, performed in the presence of 240 ng of swine DNA or 240 ng of bacterial DNA extracted from the feces of normal swine, produced a 319-bp band which was absent in reaction products derived from amplification of 300 ng of swine DNA alone (Fig. 1A). Reamplification with the internal primers C and D confirmed that the amplified product was derived from IS-intracellularis.

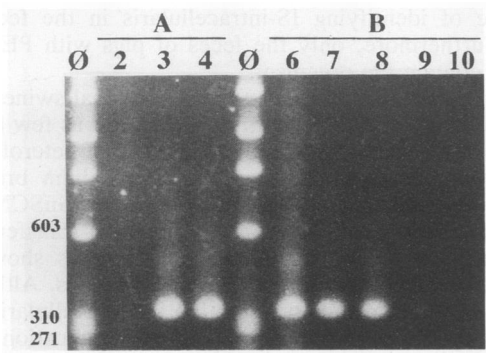


FIG. 1. (A) PCR-based detection of IS-intracellularis in infected mucosal filtrates and normal porcine tissues. Amplifications were performed with external primers (primers A and B) on samples of DNA extracted from 50 μl of homogenized tissue or infected mucosal filtrate. Three microliters of each PCR mixture was analyzed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Lanes ϕ are *Hae*III-digested ϕX174 replicative-form DNA. Results in lane 2 were obtained from 300 ng of porcine DNA; lane 3 is from infected mucosal filtrate equivalent to $2 \times 10^4$ IS-intracellularis and 240 ng of porcine DNA; lane 4 is from 240 ng of porcine DNA with the equivalent of $2 \times 10^4$ IS-intracellularis from infected mucosal filtrate and 240 ng of heterologous bacterial DNA. (B) PCR amplification of IS-intracellularis DNA extracted from infected mucosal filtrate. DNA extracted from infected mucosal filtrate was amplified by PCR with primers A and B. PCR mixtures contained DNA extracted from $10^4$ (lane 6), $10^3$ (lane 7), $10^2$ (lane 8), $10^1$ (lane 9), or $10^0$ (lane 10) IS-intracellularis as determined by indirect fluorescent-antibody assay.

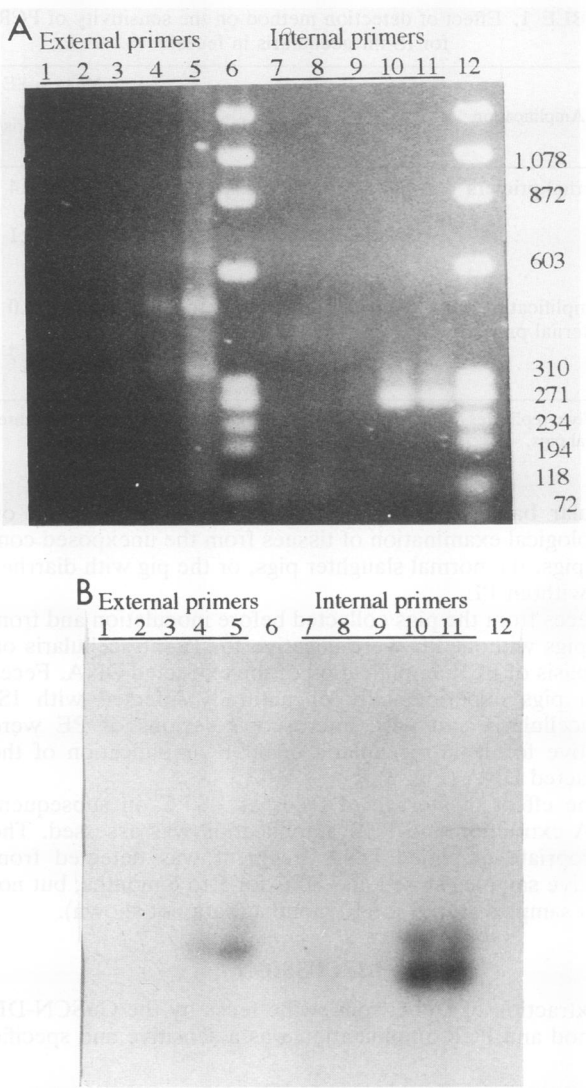


FIG. 2. Sensitivity of the PCR assay for detection of IS-intracellularis DNA from mock-infected (spiked) feces. Reaction products from fecal DNA extracts from feces spiked with PBS (lane 1) or $10^1$ (lane 2), $10^2$ (lane 3), $10^3$ (lane 4), or $10^4$ (lane 5) IS-intracellularis per g of feces were loaded in each lane. Lanes 6 and 12, *Hae*III-digested ϕX174 replicative-form DNA. One microliter of each reaction mixture was then reamplified with internal primers (primers C and D). Lanes 7 to 11 were loaded with reamplification products from feces spiked with PBS (lane 7) or $10^1$ (lane 8), $10^2$ (lane 9), $10^3$ (lane 10), or $10^4$ (lane 11) IS-intracellularis per g of feces. IS-intracellularis concentrations were determined by indirect fluorescent-antibody assay. (A) Agarose gel electrophoresis and ethidium bromide staining. (B) Southern blot and chemiluminescence detection.

The sensitivity of PCR for the detection of IS-intracellularis was determined by examination of ethidum bromide-stained agarose gels containing reaction products from infected mucosal filtrate. The 319-bp band was detected from $10^4$ to as few as $10^1$ IS-intracellularis organisms (Fig. 1B).

The sensitivity of PCR used to detect IS-intracellularis shed in the feces of infected pigs was assessed by the addition of 1-ml serial dilutions of infected mucosal filtrate in PBS to 1-g aliquots of normal feces, extraction of DNA, and amplification by PCR. DNA products of 319 bp were detected by gel electrophoresis and ethidium bromide staining following amplification of samples extracted from feces spiked with as few as $10^3$ IS-intracellularis per g of feces (Fig. 2A). Southern transfer, hybridization with digoxigenin-labeled p78, and detection by chemiluminescence verified that the band was specific for IS-intracellularis (Fig. 2B). Reamplification of the amplification products with primers C and D also confirmed that the amplification products were IS-intracellularis-specific DNA (Fig. 2A). Southern blotting often increased the sensitivity of the assay approximately 10-fold, and reamplification with internal primers C and D increased the sensitivity an additional 10-fold in five replicates of the experiment (Table 1).

PCR-based detection of IS-intracellularis shed into the feces of diseased pigs was demonstrated with fecal samples from experimentally and naturally infected pigs. Fecal samples were collected from experimentally infected pigs and an unexposed control before inoculation with PE-infected mucosa and at necropsy at 2, 4, and 5 weeks after inoculation. Feces were also collected from four pigs with PE, a pig with diarrhea but without lesions of PE, two healthy pigs selected at slaughter, and a healthy pig from an isolated herd.

DNA was extracted from the feces by the GuSCN-DE method. Experimental pigs were euthanized by intravenous injection, necropsied, and examined for microscopic lesions of PE as described above. In all pigs, the diagnosis of PE and the presence of IS-intracellularis were determined by histological examination of fixed tissue sections as described in Materials and Methods. The experimentally infected pigs had either focal or generalized infections with IS-intracellularis and microscopic proliferative lesions of PE. Pigs with naturally occurring PE had generalized microscopic proliferative lesions and IS-intracellularis infection. Neither intra-

TABLE 1. Effect of detection method on the sensitivity of PCR for IS-intracellularis in feces[a]

| Amplification | Detection method | Mean ± SE $\log_{10}$ IS-intracellularis/g of feces |
|---|---|---|
| External primers | Gel electrophoresis, ethidium bromide staining | 5 ± 1.4 |
| | Southern blotting, hybridization with p78 | 4 ± 1.1 |
| Reamplification with internal primers | Gel electrophoresis, ethidium bromide staining | 3.2 ± 1.0 |
| | Southern blotting, hybridization with p78 | 3 ± 0.7 |

[a] Five replicates of spiked feces were prepared with feces from three normal pigs.

cellular bacteria nor proliferative lesions were found on histological examination of tissues from the unexposed control pigs, the normal slaughter pigs, or the pig with diarrhea but without PE.

Feces from the pigs collected before inoculation and from the pigs without PE were negative for IS-intracellularis on the basis of PCR amplification of the extracted DNA. Feces from pigs experimentally or naturally infected with IS-intracellularis and with microscopic lesions of PE were positive for IS-intracellularis on PCR amplification of the extracted DNA (Fig. 3).

The effect of storage of feces at −80°C on subsequent DNA extraction and PCR amplification was assessed. The appropriate amplified DNA fragment was detected from positive samples stored at −80°C for 1 to 6 months, but not from samples stored for 10 months (data not shown).

## DISCUSSION

Extraction of DNA from swine feces by the GuSCN-DE method and PCR amplification was a sensitive and specific technique of identifying IS-intracellularis in the feces of swine. Furthermore, only the feces of pigs with PE were found to contain the organism.

Swine DNA or DNA extracted from normal swine feces was not amplified with primers A and B, but as few as $10^1$ IS-intracellularis organisms in the presence of heterologous DNA were amplified and detected by ethidium bromide staining. Extraction of DNA from feces by GuSCN-DE, PCR amplification, electrophoresis, Southern transfer, and hybridization with digoxigenin-labeled p78 was shown to detect $10^3$ to $10^4$ IS-intracellularis per g of feces. Although the most sensitive means of detecting IS-intracellularis was achieved by PCR amplification and reamplification with internal primers, reamplification was not necessary for the detection of IS-intracellularis in the feces of infected pigs.

Dot blot hybridization has been shown to be capable of detecting IS-intracellularis in the feces of subclinically infected pigs (8). However, detection of IS-intracellularis by use of PCR, Southern transfer, and hybridization to digoxigenin-labeled p78 was $10^3$- to $10^4$-fold more sensitive than detection by dot blot hybridization.

IS-intracellularis DNA was amplified from DNA extracted from the feces of three experimentally and four naturally infected pigs but not from feces collected before inoculation, feces of a herd mate of an age similar to those of the infected pigs held in isolation, or feces of normal slaughter pigs. Proliferative lesions and intracellular bacteria characteristic of PE were found on microscopic examination of the ileal mucosae of all pigs found to be positive for IS-intracellularis in feces by PCR. Thus, extraction of DNA from swine feces and PCR amplification of an IS-intracellularis-specific DNA sequence may be useful in the antemortem diagnosis of PE in swine.

The lack of antemortem diagnostic techniques has limited investigations of the epidemiology of PE, the prevalence of PE, the effectiveness of methods of treatment, and the economic impact of the disease. Because IS-intracellularis is always associated with the presence of PE (12), the development of this sensitive and accurate assay for its presence in swine feces promises to provide such a diagnostic test.

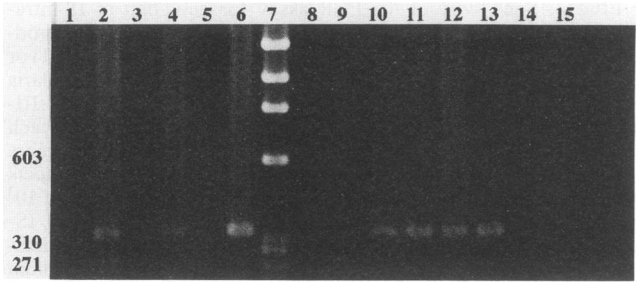


FIG. 3. Extraction and amplification of DNA from the feces of healthy pigs and pigs experimentally and naturally affected with PE. Agarose gel electrophoresis and ethidium bromide staining were used to analyze the products. DNA was extracted and amplified from feces collected from pigs before (lanes 1 and 3) and after (lanes 2, 4, and 6) experimental transmission of PE. DNA was extracted and amplified from the feces of a pig that was about the same age as the infected pigs (lane 5) and that was held as an uninfected control, a healthy pig from an isolated farm (lanes 9), and normal pigs examined at slaughter (lanes 14 and 15). DNA was also extracted from the feces of pigs with diarrhea examined at necropsy without (lane 8) and with (lanes 10, 11, 12, and 13) lesions of PE. Lane 7, *Hae*III-digested ϕX174 replicative-form DNA. Southern transfer and hybridization were also performed, with the same results (data not shown).

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