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In Situ Hybridization for the Detection and Localization of Swine *Chlamydia trachomatis*

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Abstract. Gnotobiotic piglets were inoculated intralaryngeally with swine *Chlamydia trachomatis* strain R33 or orally with swine *C. trachomatis* strain R27. Archived formalin-fixed, paraffin-embedded tissues from piglets euthanatized 4–7 days postinoculation were examined by in situ hybridization for *C. trachomatis* nucleic acid using a nonradioactive digoxigenin-labeled DNA probes that targeted specific ribosomal RNA or *omp1* mRNA molecules of the swine *C. trachomatis* strains. Positive hybridization signals were detected in bronchial epithelial cells, bronchiolar epithelial cells, pneumocytes, alveolar and interstitial macrophages, and jejunal and ileal enterocytes. Chlamydia-infected cells had a strong signal that was confined to the intracytoplasmic inclusions. Positive hybridization signals were not detected in tissue sections from an uninfected control piglet or in *C. psittaci*-infected sheep placenta. The morphology of host cells was preserved despite the relatively high temperature required in parts of the incubation procedure. The data indicate that in situ hybridization can be used to detect swine *C. trachomatis* in formalin-fixed, paraffin-embedded tissue specimens.

Key words: *Chlamydia trachomatis*; enteritis; in situ hybridization; pneumonia; swine.

Chlamydiae are gram-negative intracellular parasitic bacteria, and four species have been described. Strains of *Chlamydia trachomatis* and *C. pneumoniae* infect human beings, and strains of *C. psittaci* and *C. pecorum* are predominantly animal pathogens.²⁷ Although chlamydiae have been isolated from and/or detected in cases of pneumonia, enteritis, conjunctivitis, polyarthritides, pericarditis, perinatal mortality, and reproductive disorders in swine,^{14,17,18,21,24,26,28,29,31–33} some of these swine chlamydial strains have only recently been characterized and are believed to be *C. trachomatis* or *C. trachomatis*-like strains.^{7,13,34}

Chlamydia and chlamydia-like organisms have one or two copies of a typical eubacterial ribosomal operon that contains no tRNAs. In this operon, variable sequences in the ribosomal 16S/23S intergenic spacer and in Domain I of the 23S gene are specific for each of nine different phylogenetic groups of chlamydiae. The group containing chlamydial strains from European and American swine is closely related to human and mouse strains of *C. trachomatis*.⁷

Swine chlamydial strains R33 and R27, originally isolated from nursery pigs with pneumonia and diarrhea, respectively, are pathogens in gnotobiotic piglets.^{20,22} Both of these strains are currently classified as *C. trachomatis* or *C. trachomatis*-like chlamydiae.⁷

The objective of the present study was to develop an in situ hybridization technique using a nonradioactive digoxigenin-labeled DNA probe that targeted specific ribosomal RNA (rRNA) molecules of these swine *C. trachomatis* strains in formalin-fixed, paraffin-embedded tissue specimens.

Materials and Methods

Tissue specimens

Archived formalin-fixed, paraffin-embedded tissue specimens from gnotobiotic piglets inoculated intralaryngeally with swine *C. trachomatis* strain R33 and from gnotobiotic piglets inoculated orally with swine *C. trachomatis* strain R27 were used as test specimens.^{20,22} The piglets infected intralaryngeally with strain R33 were necropsied 7 days postinoculation, and the piglets inoculated orally with strain R27 (10⁹ colony-forming units) were necropsied 4 days postinoculation.^{20,22}

Intestinal and lung tissue specimens from an uninfected 1-day-old colostrum-deprived piglet, lung tissue from a 5-week-old conventional pig infected with *Actinobacillus pleuropneumoniae*, and intestinal tissues from a 1-week-old conventional pig infected with *Escherichia coli* served as negative tissue controls. Placenta from a ewe that had been experimentally infected with *C. psittaci* also was used as a negative tissue control.

Preparation of the *C. trachomatis*-specific probes

Probes were prepared by polymerase chain reaction (PCR) amplification of chlamydial genomic DNA from two swine strains (R33 and R27) of *C. trachomatis* grown in Vero cell monolayers.⁸ Chlamydiae were isolated from the infected cells and medium by adding Renograffin (Solvay Animal Health, Mendota Heights, MN) to a final concentration of 29%, incubating at room temperature for 10 minutes, breaking up the Vero cell DNA with a syringe using a 20–26-gauge needle, and centrifuging for 20 minutes at 11,000 rpm. The chlamydial pellets were washed three or four times in phosphate-buffered saline (PBS), resuspended in 50–100 μ l of buffer containing 50 mM dithiothreitol and 30 mM Tris/10 mM ethylenediaminetetraacetic acid (EDTA) (pH 9.0), and incubated for 1 hour at 37 C. An equal volume of 1% Nonidet P-40 and a small amount of RNase was then added. The DNA preparations were incubated at 37 C for 1 hour and then extracted with phenol/chloroform and chloroform.²³ Approximately 0.5 μ g of chlamydial DNA was used in each 100- μ l PCR. The 0.5-kb probe defined by primer 170 (5'-TCGTAACAAGGTAGCCC-3') and primer 23R (5'-TAC-TAAGATGTTTCAGTTC-3') encompasses the group-specific 16S/23S intergenic spacer and Domain I of the 23S gene. Primer 170 matches the 16S ribosomal gene sequence in all known chlamydiae (>50 strains) and a chlamydia-like organism, beginning 45 bases from the 3' end of the 16S ribosomal gene. Primer 23R complements the 23S ribosomal gene sequence in >50 strains of chlamydiae, terminating 211 bases into the 23S ribosomal gene. The ribosomal PCR products were amplified with a PCR reagent kit (Perkin Elmer, Foster City, CA) using a thermal cycler (Hybaid OmniGene Temperature Cycler, Middlesex, UK) with the following parameters: 35 cycles of 25 seconds at 95 C, 15 seconds at 50 C, and 40 seconds at 72 C and one final extension cycle of 7 minutes at 72 C. PCR amplification of 1.1 kb of the chlamydial *omp1* gene was done using primers CTU (5'-AT-GAAAAAAGCTCTTGAAATCGG-3') and CTL (5'-CAAGATTTTCTAGA(T/C)TTCAT(C/T)TTGTT-3')⁵ as described above, but the annealing conditions were 51 C for 1 minute and extension time was 1 minute. The *omp1* products encode 80% of a gene that expresses the chlamydial major outer protein.

The PCR products were extracted with phenol/chloroform and with chloroform and concentrated over microconcentrators (Microcon 100, Amicon, Beverly, MA). Several volumes of water were used to wash out the primers and to dilute them to the following concentrations: R33 ribosomal, 1.5 μ g in 27 μ l; R33 *omp1*, 3.8 μ g in 34 μ l; R27 ribosomal, 1.5 μ g in 27 μ l; R27 *omp1*, 2.6 μ g in 30 μ l. Purified PCR product was labeled by random priming with digoxigenin-dUTP using a commercial kit (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's instructions.

In situ hybridization

Paraffin-embedded sections were cut at 4 μ m, placed on Superfrost/plus slides (Fisher Scientific, Pittsburgh, PA), and stored at room temperature for in situ hybridization. Tissue sections were deparaffinized and rehydrated in PBS (pH 7.4, 0.01 M) for 5 minutes just prior to use. Deproteinization was

done in 0.2 N HCl for 20 minutes at room temperature. Tissue sections were then digested at 37 C for 20 minutes in 20 μ g/ml proteinase K (Gibco BRL, Grand Island, NY) in PBS (pH 7.4, 0.01 M) and fixed in 4% paraformaldehyde in PBS for 5 minutes. After rinsing twice in PBS, the slides tissue sections were acetylated in 30 ml of 0.1 mM triethanolamine HCl buffer (pH 8.0) to which 0.75 ml of acetic anhydride (0.25%) was added. After 5 minutes, 0.75 ml of acetic anhydride was added for an additional 5 minutes, and the tissue sections were then rinsed in 2 $\times$ standard sodium citrate (SSC; 1 $\times$ SSC is 50 mM NaCl and 15 mM sodium citrate, pH 7.0).

The tissue sections were allowed to equilibrate for 60 minutes in standard hybridization buffer that consisted of 5 $\times$ SSC, 50% deionized formamide, 2% 10 $\times$ blocking buffered solution (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine, and 0.02% sodium dodecyl sulfate. Hybridization was carried out overnight at 45 C. The digoxigenin-labeled probe (0.1 ng/ μ l) was diluted in 300 μ l of the standard hybridization buffer, heated for 5 minutes in a 95 C heating block, and then quenched on ice before application to the tissue sections. Approximately 75 ng of the digoxigenin-labeled probes was put into 70 μ l of the standard hybridization solution buffer and layered over the tissue sections. The fluid was held in place by a coverslip, and the edges were sealed with rubber cement. After overnight hybridization, tissue sections were subjected to two stringent 5-minute washes in 4 $\times$ SSC at room temperature followed by one wash in 2 $\times$ SSC for 10 minutes at 37 C. Tissue sections were then washed once in 0.2 $\times$ SSC containing 60% formamide for 10 minutes at 37 C, twice in 2 $\times$ SSC for 5 minutes at room temperature, twice in 0.2 $\times$ SSC for 5 minutes at room temperature, and once in buffer I (100 mM maleic acid, 150 mM NaCl) (pH 7.5) for 5 minutes at room temperature.

Tissue sections were incubated with antidigoxigenin conjugated with alkaline phosphatase (Boehringer Mannheim) diluted 1:200 in 0.1 M Tris-HCl (pH 7.4) and 0.15 M NaCl with 1% blocking reagent (Boehringer Mannheim) for the detection of hybrids. After three washes in buffer I, substrate consisting of nitroblue tetrazolium (NBT) and 5-bromocresyl-3-indolylphosphate (BCIP) was layered over the sections. Color was allowed to develop for 5–8 hours in the dark, and the reaction was stopped by dipping the slides briefly in Tri-EDTA (10 mM Tris-HCl, 1 mM EDTA) buffer (pH 8.0). Tissue sections were counterstained with 0.5% methyl green, washed with distilled water for 1 minute, and then allowed to dry completely.

Results

Chlamydia trachomatis strain R33 was detected in the lungs of all intralaryngeally inoculated pigs with the probe made using the R33 strain. There was no difference in intensity of hybridization signals between rRNA and *omp1* mRNA probes to detect *C. trachomatis* strain R33 in lung tissues. The morphology of host cells was preserved despite the relatively high temperature required in parts of the incubation procedure. Positive cells typically were dark brown to black, and there was no background staining. Generally, individ-

Fig. 1. Lung; piglet necropsied 7 days postinoculation. Chlamydial nucleic acid (dark brown reaction) appears in scattered bronchiolar epithelium. In situ hybridization; rRNA probe, NBT/BCIP, methylgreen counterstain. Bar = 55 μ m.

Fig. 2. Lung; piglet necropsied 7 days postinoculation. Chlamydial nucleic acid (dark brown reaction) appears in scattered thickened alveolar septa. In situ hybridization; *omp1* mRNA probe, NBT/BCIP, methylgreen counterstain. Bar = 55 μ m.

ual positive cells were scattered; however, occasionally there was a focus in which several positive cells were aggregated. Bronchial and bronchiolar epithelial cells (Fig. 1), pneumocytes, and alveolar macrophages all had positive hybridization signals. An especially strong hybridization signal was detected in interstitial and alveolar macrophages in the thickened alveolar septa and spaces (Fig. 2).

Chlamydia trachomatis strain R27 was detected in the jejunum and ileum of all orally inoculated pigs with the probe made using the R27 strain. There was no difference in intensity of hybridization signals between rRNA and *omp1* mRNA probes to detect *C. trachomatis* strain R27 in intestinal tissues. There were strong hybridization signals in the cytoplasm of enterocytes lining the apical portions of villi, typically in areas of vacuolated enterocytes and moderate to severe villus atrophy (Fig. 3). There was no hybridization signal in epithelial cells lining the crypts of Lieberkühn. There were no hybridization signals in the lung and intestinal tissues from the control pigs or in the sheep placenta infected with *C. psittaci*.

Discussion

This is the first demonstration of swine chlamydial nucleic acid by in situ hybridization in formalin-fixed, paraffin-embedded tissue specimens using a nonradioactive labeled DNA probe. *C. trachomatis* antigen has been detected in bronchial and bronchiolar epithelial cells, pneumocytes, alveolar and interstitial macrophages, and jejunal and ileal villus enterocytes of swine using polyclonal-antibody-based immunohistochemical procedures.^{20,22,34} However, use of DNA probes and in situ hybridization eliminates possibilities for error caused by antigenic cross-reactivity or by the alteration of binding sites during tissue processing. Formalin fixation can denature antigens, which can lead to false-negative results.^{10,19} In contrast to immunohistochemistry, in situ hybridization is less susceptible to structural alteration caused by fixation.⁹

The R33 and R27 swine ribosomal probe sequences differ from the ribosomal sequences of other swine strains by 0.4–3%, and they differ from human and mouse strains of *C. trachomatis* by 3–5%. These ribosomal probes differ from all strains of *C. psittaci*, *C. pneumoniae*, and *C. pecorum* by 15–18%. They also differ from the most closely related chlamydia-

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Fig. 3. Jejunum; piglet necropsied 4 days postinoculation. Chlamydial nucleic acid (dark brown reaction) is present in epithelium covering moderately atrophic villi. In situ hybridization; rRNA probe, NBT/BCIP, methylgreen counterstain. Bar = 80 μ m.

like sequence by 50%, with most of the conservation located in the 16S and 23S portions of the PCR product. The R33 and R27 *omp1* probes differ 24% from each other, and they differ 16–24% from other swine strains. The swine *omp1* probes differ 26–31% from human and mouse *C. trachomatis* strains, and they differ 42–52% from all other strains of *Chlamydia* (unpublished data). Thus, a complementary DNA probe from swine *C. trachomatis* could be used for the specific detection of *C. trachomatis* in swine tissue specimens.

Currently, the diagnosis of chlamydial infection is based primarily upon the isolation of the organism in either HeLa or McCoy cells. However, the sensitivity of such isolations are typically 90–95% compared with that of other methods.¹⁵ Direct fluorescent antibody tests and enzyme immunoassays using monoclonal antibodies have been described and compared with culture methods for the detection and identification of *C. trachomatis*.²⁵ However, none of the immunoassays have been highly sensitive or specific.²⁵ PCR procedures³⁰ and molecular hybridization screening systems that target chlamydial nucleic acids⁴ can detect those molecules with high specificity and sensitivity, but they can give no information regarding the specific location of *C. trachomatis* in tissues. In situ hybridization can be useful in addressing all these questions; it can be used to identify the types of infected host cells with high specificity and sensitivity.

Probes used in previous in situ hybridization assays targeting *C. trachomatis* have consisted of the chlamydial plasmid, cloned fragments of the chlamydial chromosome, or total chlamydial chromosomal DNA. Various methods of probe labeling, including radioactivity and biotinylation, have been used in to investigate human chlamydial infections.^{3,6,11,12,16} However, all of these methods are inadequate for the examination of clinical samples.² Furthermore, chlamydiae lacking a specific plasmid have been described,¹ and the use of such DNA sequences as probes would be problematic.

This new in situ hybridization system is highly specific and employs a nonradioactively labeled DNA probe that is targeted to specific chlamydial rRNA sequences. This system can be used with formalin-fixed, paraffin-embedded tissue specimens and can distinguish *C. trachomatis* from *C. psittaci* infection.

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