NOTE *Pathology*

Digoxigenin-Labeled *In Situ* Hybridization for the Detection of *Streptococcus suis* DNA in Polyserositis and a Comparison with Biotinylated *In Situ* Hybridization

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(Received 22 April 2013/Accepted 14 August 2013/Published online in J-STAGE 28 August 2013)

ABSTRACT. The objective of this study was to develop digoxigenin-labeled *in situ* hybridization (ISH) for the detection of *Streptococcus suis* in naturally infected pigs with polyserositis and to compare it with biotinylated ISH. Digoxigenin-labeled hybridization signals for *S. suis* were observed in cells that had infiltrated the fibrous polyserositis and microcolonies in the blood vessels. Mock hybridization showed no hybridization signals for endogenous digoxigenin. Biotinylated hybridization signals for *S. suis* were observed in cells that had infiltrated the fibrous polyserositis. However, similar hybridization signals were also observed in the fibrous inflammatory area using mock hybridization for endogenous biotin. The present study demonstrated that digoxigenin-labeled ISH is a valuable diagnostic tool for specific detection of *S. suis* in polyserositic tissues without nonspecific reactions compared with biotinylated ISH.
KEY WORDS: *In situ* hybridization, Polyserositis, *Streptococcus suis*.

doi: 10.1292/jvms.13-0204; *J. Vet. Med. Sci.* 76(1): 109–112, 2014

Polyserositis is an economically important disease that has been recognized as a general inflammation of serous membranes, such as the pleura, pericardium and peritoneum. Polyserositis is mainly caused by *Haemophilus parasuis*, *Streptococcus suis* and *Mycoplasma hyorhinis* [1, 7, 17]. Among these three pathogens, *H. parasuis* has been described as the most common etiological agent, followed by *S. suis* and *M. hyorhinis,* in Korea [11]. Precise diagnosis of polyserositis has depended heavily on isolation of the etiological agent, followed by examination of its biochemical and morphological properties. Culture of these bacterial pathogens can be relatively insensitive, especially in chronic cases with polyserositis [11]. Recently, multiplex polymerase chain reaction (PCR) was developed for the detection and differentiation of these pathogens in formalin-fixed paraffin-embedded (FFPE) tissues [11]. However, detection of these organisms by PCR only may not enable a definite diagnosis of polyserositis, because *H. parasuis*, *S. suis* and *M. hyorhinis* are commonly isolated from normal healthy pigs [2, 6, 16]. Alternatively, *in situ* hybridization (ISH) is useful to avoid misinterpretation of PCR results. Digoxigenin-labeled ISH has been reported for the detection of *H. parasuis* and *M. hyorhinis* in polyserositic tissues [10, 12]. Although *S. suis* DNA was detected in FFPE tissues by biotinylated ISH, this technique produces some degrees of false-positive results because of endogenous biotin in porcine tissues [4, 5]. Hence, the objective of this study was to develop digoxigenin-labeled ISH for detection of *S. suis* DNA in FFPE tissues in pigs with polyserositis.

Twenty pigs were selected from 24 in which *S. suis* infection was diagnosed on the basis of bacterial isolation and microscopic lesions, such as fibrinous pericarditis, pleuritis and peritonitis. Of the 20 cases, 7 different serotypes were identified by the coagglutination technique [8]: serotype 2 (2 cases), serotype 3 (4 cases), serotype 4 (4 cases), serotype 8 (2 cases), serotype 16 (1 case), serotype 22 (1 case) and serotype 33 (2 cases). In addition, 2 untypable and 2 autoagglutinating strains were recovered in the last 4 cases [13]. The 16S rRNA genes of 20 isolates were sequenced and confirmed as *S. suis* as previously described [3]. Five cardiac sections with pericarditis from different pigs naturally infected with *H. parasuis* or *M. hyorhinis* were used to provide further control materials [10, 12]. Two additional sections with mastitis from cows naturally infected with *S. agalactiae* and *S. parauberis* were used as control materials.

A 228 base pair DNA fragment from 16S rRNA of *S. suis* serotype 2 (SNUVP 650099) generated by the PCR was used as a probe. The probe sequence for *S. suis* used in the present study has more than 90% homology with the 16S rRNA gene sequence of some other streptococcal species in

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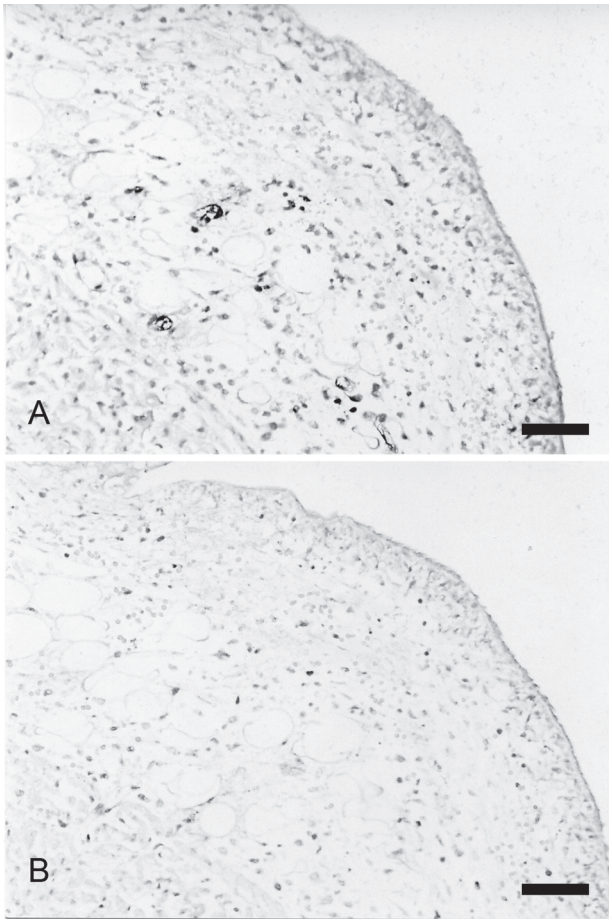

Fig. 1. Consecutive serial sections of pericardium from a pig naturally infected with *Streptococcus suis* serotype 2. Digoxigenin-labeled hybridization signals for *S. suis* were detected in the severe fibrinous inflammatory area (A). Pretreatment with DNase I eliminated digoxigenin-labeled hybridization signals from the section (B). Bar=100 μm.

BLAST search results (http://www.ncbi.nlm.nih.gov/blast/; *S. agalactiae,* 93.0%; *S. parauberis,* 91.7%).

The forward and reverse primers were 5′-AACGCTGAAGTCTGGTGCTT-3′ (nucleotides 38–57) and 5′-TGTATCGATGCCTTGGTGAG-3′ (nucleotides 246–265), respectively [11]. The primers were determined by BLAST 2.2.22+ (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to be highly specific for *S. suis*. No other sequences of *S. agalactiae* and *S. parauberis* completely matched the designed primers.

The PCR for the 16S rRNA gene of *S. suis* was carried out as previously described [11]. PCR products were purified with a 30-kD cutoff membrane filter. Nucleotide sequencing was performed on the purified PCR products. Purified PCR products were labeled by random priming with digoxigenin-dUTP (Boehringer Mannheim, Indianapolis, IN, U.S.A.) or biotin-dUTP (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Six serial sections (4 μm) were mounted on positively charged slides (Superfrost/Plus slides, Erie Scientific Co., Portsmouth, NH, U.S.A.) and then prepared from each tissue, two being further processed for ISH using an *S. suis* probe with and without DNase I (Boehringer Mannheim) treatment and four being prepared for ISH using a *H. parasuis* and *M. hyorhinis* probe with and without DNase I treatment. Just before use, they were dewaxed in xylene, rehydrated in phosphate-buffered saline (PBS; pH 7.4, 0.01 M) for 5 min and deproteinized with 0.2 N HCl for 20 min at room temperature. They were then digested at 37°C for 20 min in PBS containing 200 μg/m*l* proteinase K (Gibco BRL, Grand Island, NY, U.S.A.). For each tissue examined, a serial section was treated with DNase I at 0.1 unit/m*l* in 10 mM Tris-HCl (pH 7.4) for 30 min at 37°C to remove target DNA as a specificity control. After digestion, tissues were fixed in 4% paraformaldehyde in PBS for 10 min. After rinsing with PBS twice, the slides were acetylated in 300 m*l* of 0.1 mM triethanolamine-HCl buffer (pH 8.0) to which 0.75 m*l* of acetic anhydride (0.25%) had been added. After 5 min, a further 0.75 m*l* of acetic anhydride was added, and 5 min later, the slides were rinsed in 2X saline sodium citrate (SSC; 1X SSC contains 50 mM NaCl and 15 mM sodium citrate, pH 7.0).

Hybridization was carried out overnight at 45°C. The digoxigenin-labeled (or biotinylated) probe was diluted to 1 *n*g/μ*l* in standard hybridization buffer consisting of 2X SSC containing 50% deionized formamide, 10 mg salmon sperm DNA (Oncor, Gaithersburg, MD, U.S.A.), 0.02% sodium dodecyl sulphate (SDS), 1X Denhart's solution and 12.5% dextran sulphate. Approximately 70 *n*g of digoxigenin-labeled (or biotinylated) probe contained in standard hybridization buffer (70 μ*l*) was layered over the section. Fluid was held in place by a coverslip (the edges of which were sealed with rubber cement) and heated for 10 min in a 95°C heating block. After overnight hybridization, sections were thoroughly washed, twice in 4X SSC for 10 min at room temperature, twice in 2X SSC for 10 min at 45°C, twice in 2X SSC for 10 min at room temperature, twice in 0.2X SSC for 10 min, once in maleic acid buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5) for 5 min and once in 1X blocking reagent (Boehringer Mannheim) for 40 min at room temperature. Hybridization signals for digoxigenin-labeled ISH were visualized by anti-digoxigenin conjugated with alkaline phosphatase (Boehringer Mannheim) as previously described [10]. Hybridization signals for biotinylated ISH were visualized by streptavidin-conjugated alkaline phosphatase [14].

Mock hybridization was carried out to evaluate problems encountered with endogenous biotin. Tissue sections were mock hybridized in hybridization buffer only. Otherwise, the pre- and post-hybridization procedures are the same as for routine ISH. Mock hybridization signals were also visualized by anti-digoxigenin or streptavidin conjugated with alkaline phosphatase.

ISH produced a distinct positive signal for the *S. suis* gene in the polyserositis. The intensity and extent of labeling for *S. suis* were detected in the fibrous inflammatory area of polyserositis in various tissues: the pericarditis (Fig. 1A),

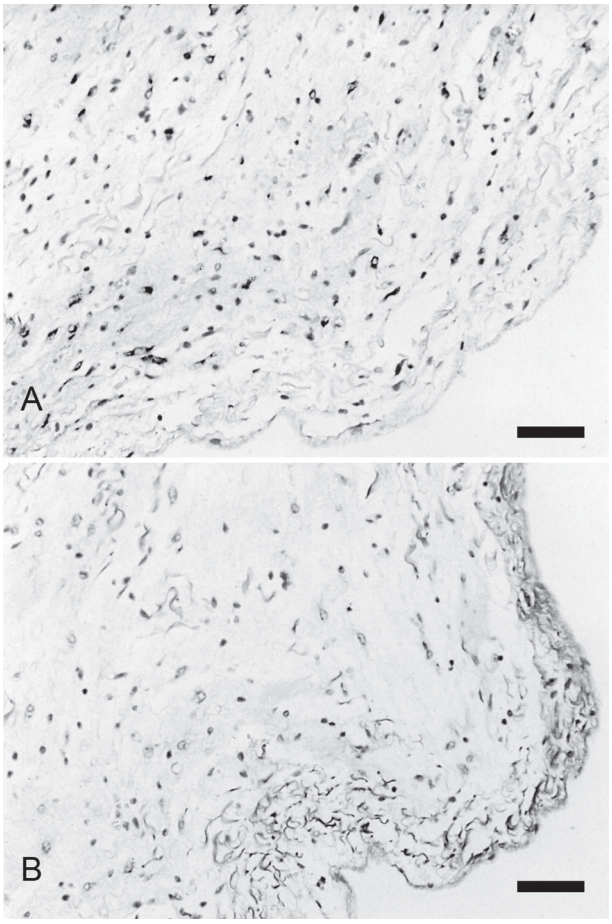

Fig. 2. Consecutive serial sections of pericardium from a pig naturally infected with *Streptococcus suis* serotype 4. Biotinylated hybridization signals for *S. suis* were detected in the severe fibrinous inflammatory area (A). Mock hybridization signals for endogenous biotin were also observed in the section (B). Bar=100 μm.

pleuritis and peritonitis. Hybridization signals were detected primarily in cells that had infiltrated the fibrinous polyserositis. Identification of the cell types containing the *S. suis* 16S rRNA gene was occasionally difficult. Digoxigenin-labeled hybridization signals for *S. suis* were observed in cells that had infiltrated the fibrous polyserositis and microcolonies in the blood vessels from the 20 samples naturally infected with *S. suis* (Fig. 1A). There was no difference in signal intensity among the 20 cases caused by different serotype strains. Pretreatment with DNase I eliminated hybridization signals from the 20 samples naturally infected with *S. suis* (Fig. 1B). Mock hybridization showed no hybridization signals for endogenous digoxigenin. Biotinylated hybridization signals for *S. suis* were observed in cells that had infiltrated the fibrous polyserositis (Fig. 2A). However, positive signals were also observed in the fibrous inflammatory area using mock hybridization for endogenous biotin (Fig. 2B).

Sections of heart with fibrinous pericarditis from the pigs naturally infected with *H. parasuis* and *M. hyorhinis* showed no hybridization signals for *S. suis* using the digoxigenin-labeled probe for *S. suis*. Moreover, the digoxigenin-labeled probes for *H. parasuis* and *M. hyorhinis* were consistently negative in the fibrous inflammatory area of streptococcal polyserositis observed in the lung, heart, spleen and liver. No hybridization signals for *S. suis* using the digoxigenin-labeled probe for *S. suis* were detected in the sections of mammary glands from the cow naturally infected with S *agalactiae* and *S. parauberis*.

The present study demonstrated that *S. suis* can be detected and differentiated from *H. parasuis* and *M. hyorhinis* in FFPE tissue specimens of infected pigs by means of a digoxigenin-label DNA probe. ISH using a biotinylated probe had been reported for the detection of *S. suis* in formalin-fixed tissues [14]. However, biotin is an endogenous molecule of living cells associated with carboxylases and plays a key role in many reactions, mainly in the liver and kidney [19]. Endogenous biotin was detected widely in many tissues of pigs, whereas digoxigenin is exclusively present in digitalis plants (*Digitalis purpurea* or *D. lantana*) as a secondary metabolite [4, 5]. Hence, the major advantage of digoxigenin-labeled probes is elimination of false-positive results when this probe is used, because endogenous biotin may sometimes react with avidin or streptavidin reagents or anti-biotin antibodies used as components of the detection system.

Although it has been previously reported that reference strains of serotypes 22 and 33 may belong to a species different from *S. suis* [18], 16S rRNA sequencing of the three strains (one from serotype 22 and two from serotype 33) included in this study showed they are in fact *S. suis*. We do not know why there is a discrepancy between serotyping and 16S rRNA sequence analysis. However, it could be due to some cross-reactions in the coagglutination test; in previous research, antiserum against capsular serotype 2 reacted with the antigen of capsular serotype 22 [8], antiserum against capsular serotype 33 reacted with the antigen of capsular serotype 9, and antiserum against capsular serotypes 9 and 11 reacted with antigens of serotype 33 [9]. These results suggest that serotypes 22 and 33 identified by coagglutination may be serotypes 2 and 9, respectively. Further studies should be done in the future to elucidate the discrepancy in results between the 2 tests.

Pigs in which only *S. suis* was isolated showed suppurative exudation that was more extensive than that associated with *H. parasuis* [15]. However, histopathological observation alone is not able to differentiate *S. suis* infection from *H. parasuis* and *M. hyorhinis* infection. ISH provides cellular details and the histological architecture so that a small number of *S. suis*-positive signals and lesions may be observed in the same section. Therefore, it is a valuable diagnostic tool, especially when it is necessary to distinguish *S. suis* from *H. parasuis* and *M. hyorhinis* in FFPE tissues.

ACKNOWLEDGMENTS. This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries, Republic of Korea. The research was also supported by Brain

Korea 21 PLUS Program for Veterinary Science and contract research funds of the Research Institute for Veterinary Science (RIVS) from the College of Veterinary Medicine.

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