**NOTE** Pathology

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

Ikjae KANG1)**, Hwi Won SEO1)**, Changhoon PARK1), Yeonsu OH1), Jeewon LEE2), Ok Heui YOU3), Sung-Hoon KIM5), Marcelo GOTTSCHALK4) and Chanhee CHAE1)**

1)Department of Veterinary Pathology, College of Veterinary Medicine, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151–742, Republic of Korea
2)Dong Bang Co., Ltd., Dogok-Dong 517–8, Gangam-gu, Seoul 135–272, Republic of Korea
3)College of Oriental Medicine, Kyunghee University, 1 Hoegi-dong, Dongdaemun-Gu, Seoul 130–701, Republic of Korea
4)Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine, Faculty of Veterinary Medicine, University of Montreal, Quebec, Canada

(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 polyserotic tissues without nonspecific reactions compared with biotinylated ISH.

KEY WORDS: In situ hybridization, Polyserositis, *Streptococcus suis*.

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 polyserotic 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...
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′-TGATATCGATGCCTTGGTGAG-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/ml 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/ml 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 ml of 0.1 mM triethanolamine-HCl buffer (pH 8.0) to which 0.75 ml of acetic anhydride (0.25%) had been added. After 5 min, a further 0.75 ml 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 ng/µl 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 ng of digoxigenin-labeled (or biotinylated) probe contained in standard hybridization buffer (70 µl) 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),
pleuritis and peritonitis. Hybridization signals were detected primarily in cells that had infiltrated the fibrous polyserositis. Identification of the cell types containing the \textit{S. suis} 16S rRNA gene was occasionally difficult. Digoxigenin-labeled hybridization signals for \textit{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 \textit{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 \textit{S. suis} (Fig. 1B). Mock hybridization showed no hybridization signals for endogenous digoxigenin. Biotinylated hybridization signals for \textit{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 fibrous pericarditis from the pigs naturally infected with \textit{H. parasuis} and \textit{M. hyorhinis} showed no hybridization signals for \textit{S. suis} using the digoxigenin-labeled probe for \textit{S. suis}. Moreover, the digoxigenin-labeled probes for \textit{H. parasuis} and \textit{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 \textit{S. suis} using the digoxigenin-labeled probe for \textit{S. suis} were detected in the sections of mammary glands from the cow naturally infected with \textit{S. agalactiae} and \textit{S. parauberis}.

The present study demonstrated that \textit{S. suis} can be detected and differentiated from \textit{H. parasuis} and \textit{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 \textit{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 (\textit{Digitalis purpurea} or \textit{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 \textit{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 \textit{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 \textit{S. suis} was isolated showed suppurrative exudation that was more extensive than that associated with \textit{H. parasuis} [15]. However, histopathological observation alone is not able to differentiate \textit{S. suis} infection from \textit{H. parasuis} and \textit{M. hyorhinis} infection. ISH provides cellular details and the histological architecture so that a small number of \textit{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 \textit{S. suis} from \textit{H. parasuis} and \textit{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

![Image](image_url)
REFERENCES

1. Aragon, V., Segales, J. and Oliveira, S. 2012. Glasser’s disease. pp. 760–769. In: Diseases of Swine, 10th ed. (Zimmerman, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J. and Stevenson, G. W. eds.), Blackwell Publishing, West Sussex, U.K.

2. Brown, C. C., Baker, D. C. and Baker, I. K. 2007. Alimentary tract. pp. 779–797. In: Diseases of Swine, 10th ed. (Zimmerman, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J. and Stevenson, G. W. eds.), Blackwell Publishing, West Sussex.

3. Chatellier, S., Harel, J., Zhang, Y., Gottschalk, M., Higgins, R., Devriese, L. A. and Brousseau, R. 1998. Phylogenetic diversity of Streptococcus suis strains of various serotypes as revealed by 16S rRNA gene sequence comparison. Int. J. Syst. Bacteriol. 48: 581–589. [Medline] [CrossRef]

4. Chevalier, J., Yi, J., Michel, O. and Tang, X. M. 1997. Biotin and digoxigenin as labels for light and electron microscopy in situ hybridization probes: where do we stand? J. Histochem. Cytochem. 45: 481–491. [Medline] [CrossRef]

5. Cooper, K. M., Kennedy, S., McConnell, S. and Kennedy, D. G. 1997. An immunohistochemical study of the distribution of biotin in tissues of pigs and chickens. Res. Vet. Sci. 63: 219–225. [Medline] [CrossRef]

6. Flores, J. L., Higgins, R., D’Allaire, S., Charette, R., Boudreau, M. and Gottschalk, M. 1993. Distribution of the different capsular types of Streptococcus suis in nineteen swine nurseries. Can. Vet. J. 34: 170–171. [Medline]

7. Gottschalk, M. 2012. Streptococcosis. pp. 841–855. In: Diseases of Swine, 10th ed. (Zimmerman, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J. and Stevenson, G. W. eds.), Blackwell Publishing, West Sussex.

8. Gottschalk, M., Higgins, R., Jacques, M., Mittal, M. R. and Henrichsen, J. 1989. Description of 14 new capsular types of Streptococcus suis. J. Clin. Microbiol. 27: 2633–2636. [Medline]

9. Higgins, R., Gottschalk, M., Boudreau, M., Lebrun, A. and Henrichsen, J. 1995. Description of six new capsular types (29–34) of Streptococcus suis. J. Vet. Diagn. Invest. 7: 405–406. [Medline] [CrossRef]

10. Jung, K. and Chae, C. 2004. In-situ hybridization for the detection of Haemophilus parasuis in naturally infected pigs. J. Comp. Pathol. 130: 294–298. [Medline] [CrossRef]

11. Kang, I., Kim, D., Han, K., Seo, H. W., Oh, Y., Park, C., Lee, J., Gottschalk, M. and Chae, C. 2012. Optimized protocol for multiplex nested polymerase chain reaction to detect and differentiate Haemophilus parasuis, Streptococcus suis, and Mycoplasma hyorhinis in formalin-fixed, paraffin-embedded tissues from pigs with polyserositis. Can. J. Vet. Res. 76: 195–200. [Medline]

12. Kim, B., Lee, K., Han, K., Kim, D., Ha, Y., Kim, C. H., Oh, Y., Kang, I., Lee, J. and Chae, C. 2010. Development of in situ hybridization for the detection of Mycoplasma hyorhinis in formalin-fixed paraffin-embedded tissues from naturally infected pigs with polyserositis. J. Vet. Med. Sci. 72: 1225–1227. [Medline] [CrossRef]

13. Kim, D., Han, K., Oh, Y., Kim, C. H., Kang, I., Lee, J., Gottschalk, M. and Chae, C. 2010. Distribution of capsular serotypes and virulence markers of Streptococcus suis isolated from pigs with polyserositis in Korea. Can. J. Vet. Res. 74: 314–316. [Medline]

14. Madsen, L. W., Boye, M. and Jensen, H. E. 2001. An enzyme-based in situ hybridisation method for the identification of Streptococcus suis. APIMS 109: 665–669. [Medline] [CrossRef]

15. Reams, R. Y., Glickman, L. T., Harrington, D. D., Bowersock, T. L. and Thacker, H. L. 1993. Streptococcus suis infection in swine: a retrospective study of 256 cases. Part I. Epidemiologic factors and antibiotic susceptibility patterns. J. Vet. Diagn. In- vest. 5: 363–367. [Medline] [CrossRef]

16. Smart, N. L., Mminiats, O. P., Rosendal, S. and Friendship, R. M. 1989. Glasser’s disease and prevalence of subclinical infection with Haemophilus parasuis in swine in Southern Ontario. Can. Vet. J. 30: 339–343. [Medline]

17. Thacker, E. L. and Christopher, M. F. 2012. Mycoplasmosis. pp. 779–797. In: Diseases of Swine, 10th ed. (Zimmerman, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J. and Stevenson, G. W. eds.), Blackwell Publishing, West Sussex.

18. Tien, H. T., Nishibori, T., Nishitani, Y., Nomoto, R. and Osawa, R. 2013. Reappraisal of the taxonomy of Streptococcus suis serotypes 20, 22, 26, and 33 based on DNA-DNA homology and sodA and recN phylogenies. Vet. Microbiol. 162: 842–849. [Medline] [CrossRef]

19. Varma, V. A., Cerjan, C. M., Abbott, K. L. and Hunter, S. B. 1994. Non-isotopic in situ hybridization method for mitochondrial in oncocytes. J. Histochem. Cytochem. 42: 273–276. [Medline] [CrossRef]