Segalés J, Ramos-Vara JA, Duran CO, et al. Diagnosing infectious diseases using in situ hybridization. *Swine Health Prod.* 1999;7(3):125–128.

Diagnosing infectious diseases using in situ hybridization

Joaquim Segalés, DVM, PhD; José A. Ramos-Vara, DVM, PhD, Diplomate ECVP; C. Oliver Duran, DVM, MRCVS, PhD; Amy Porter; Mariano Domingo, DVM, PhD, Diplomate ECVP

Summary

This note describes the foundations, methodology, principles, advantages and disadvantages of using in situ hybridization to diagnose swine infectious diseases. In situ hybridization (ISH) relies on the detection of complementary sequences of nucleic acids present in the tissue using labeled nucleic acid sequences (probes) specific for the infectious organism. Hybridization occurs between complementary purine and pyrimidine bases of nucleic acids. The hybridization of segments of nucleic acids is revealed by an enzymatic reaction or the color emission of a fluorochrome. As with immunohistochemistry, the specificity and sensitivity of ISH makes it an excellent alternative to more complex, expensive, and time-consuming laboratory procedures such as virus isolation or microbiological culture for the diagnoses of swine infectious diseases.

Keywords: swine, diagnostics, in situ hybridization

Received: May 21, 1998
Accepted: January 4, 1999

In situ hybridization (ISH) is a technique used to diagnose swine diseases. Although ISH was developed in the late 1960s,[1–2] until recently it was not widely used to diagnose animal diseases. In situ hybridization shares some advantages with immunohistochemistry (IHC), such as low cost and high sensitivity. Although the fundamentals of ISH are different from those of IHC, these complementary techniques bridge the gap between morphologic, immunologic, and molecular biology techniques. The goal of ISH is to detect the infectious agent by demonstrating the presence of specific nucleic acid segments of an infectious agent in the sample. In this diagnostic note, we will describe the scientific basis of ISH, as well as its methodology, advantages, disadvantages, and applications for diagnosing swine infectious diseases.

JS, MD: Department of Animal Pathology and Production, School of Veterinary Medicine, 08193 Bellaterra, Barcelona, Spain; JAR-V: University of Missouri; COD, AP: Michigan State University

Reprint requests to: Veterinary Medical Diagnostic Laboratory, University of Missouri, PO Box 6023, Columbia, Missouri 65205, Email: RAMOSJ@MISSOURI.EDU

This diagnostic note has been refereed.

This is the last in a three-part series reviewing new diagnostic techniques.

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The basics of in situ hybridization

Nucleic acids (DNA, RNA) comprise the genomic material of living structures. DNA is composed of one or two complementary strands, and RNA of a single strand. Each nucleic acid strand is a linear (or, in a few cases, circular), unbranched polymer consisting of a sugar-phosphate backbone with a nucleotide base (purine or pyrimidine) linked to each sugar residue.[3] Both strands of nucleic acid are linked by hydrogen bonds. Purine bases (adenine, guanine) will bind to complementary pyrimidine bases (thymine and cytosine for DNA and uracil and cytosine for RNA). In situ hybridization relies on the principle that specific sequences of single-stranded cell- and tissue-bound RNA and DNA will hybridize with single-stranded labeled probes of complementary sequence.[3,4] Because every infectious organism has unique segments of DNA or RNA that are not found in other organisms, cells, or tissues, ISH can localize single-copy genes and mRNA transcripts in samples with fewer than 10 copies per cell present.[5]

In situ hybridization procedure

Collecting samples

Careful sample collection is critical, especially if RNA sequences are tested. Tissues have enzymes, including some called RNA-ases that destroy RNA molecules. This activity is more intense after the animal's death or after the tissue has been removed from a living body (enzymatic activity is still present but no new RNA is formed). Therefore, the sooner the tissue is collected and preserved, the better. Fixation stops the activity of these enzymes and hence ceases postmortem decomposition.

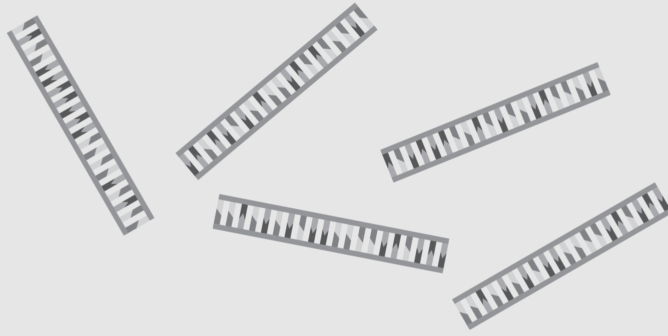
Preserving the tissues

The sensitivity of the ISH reaction is higher in frozen samples than in fixed samples,[6] but it is not advisable to perform ISH on frozen samples when appropriate equipment is lacking or there is a delay before the procedure can be performed. The routine (all purpose) tissue fixative is 10% neutral buffered formalin. Formalin adequately preserves tissue morphology and provides good retention of the nucleic acids, but reduces tissue permeability to the specific nucleic acid probe. Loss of detection of segments of nucleic acids can be related to fixation time and the length of the target nucleic acid sequence.[7]

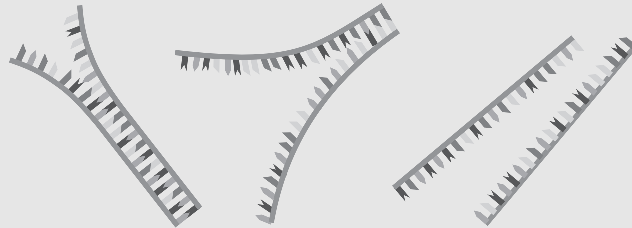
Formalin-fixed and paraffin-embedded tissues that have been archived for many years can still be used for retrospective studies with ISH.

Figure 1

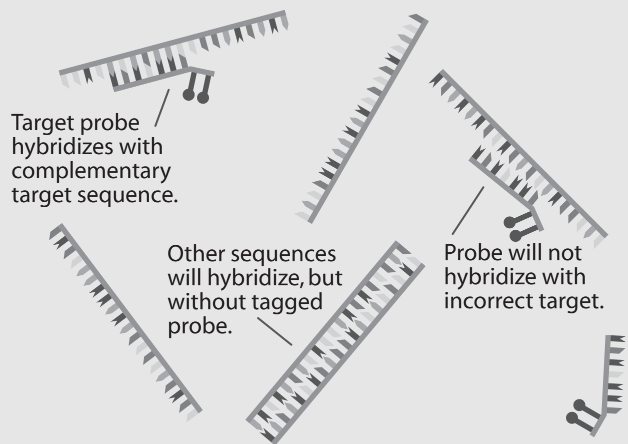
Various double-stranded nucleic acid chain "targets" are present in possibly infected tissue sample.



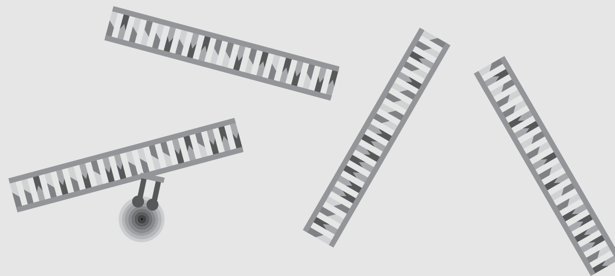
Denaturation process splits double-stranded chains into single strands, exposing nucleic-acid bases.



Tagged probe will hybridize only with complementary base sequence found in a specific genome.



Tags of probes remaining in tissue are identified with stain. Presence of stain indicates presence of pathogen.



Hybridization tagging process

Processing the samples

For diagnostic purposes, tissues for ISH are processed as for conventional histopathology.

In situ hybridization techniques

Three components are essential in any ISH technique (Figure 1):

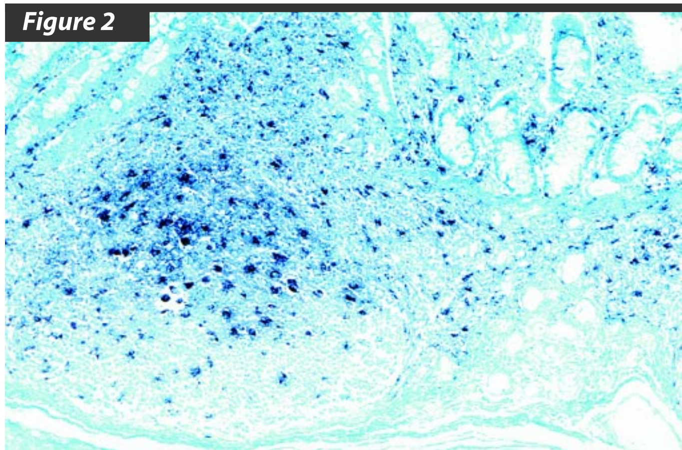
1) a nucleic acid template (DNA or RNA) within the tissue to be tested (target nucleic acid);

2) a labeled nucleic acid probe (usually an oligonucleotide which is a short chain of specifically ordered nucleotide bases[8] or segments of RNA or double-stranded DNA[5,9] specific for the organism to be detected); and

3) a detection system to demonstrate the hybridization reaction.

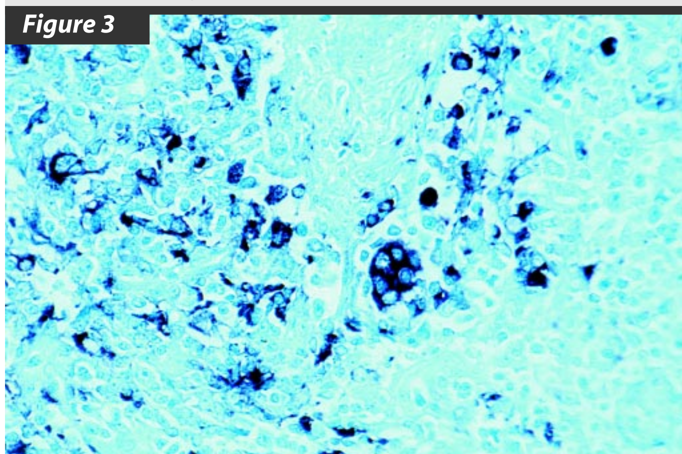
The duration of this test varies, but usually is 30–90 minutes.

Figure 2



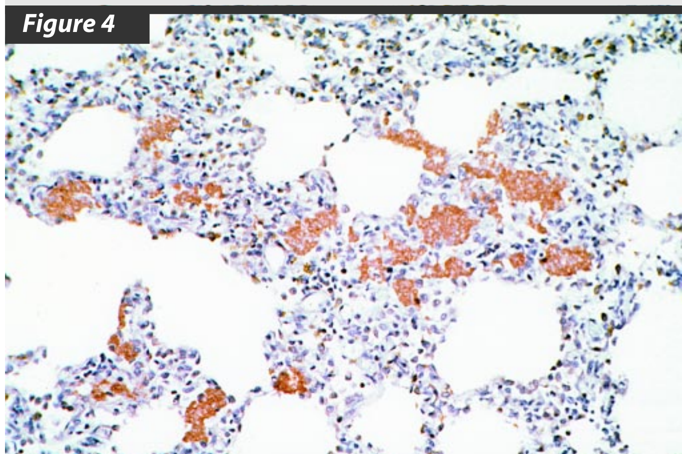
Lymph node. Numerous macrophages and multinucleated giant cells stained in blue contain specific sequences of nucleic acid for porcine circovirus. In situ hybridization. Anti-digoxigenin-alkaline phosphatase method. Fast green counterstain.

Figure 3



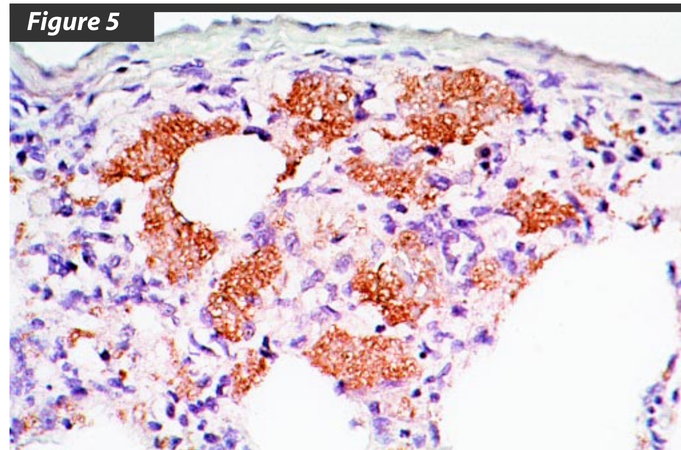
Ileum. Nucleic acid for porcine circovirus is demonstrated in blue in numerous histiocytic and lymphoid cells. Anti-digoxigenin-alkaline phosphatase method. Fast green counterstain.

Figure 4



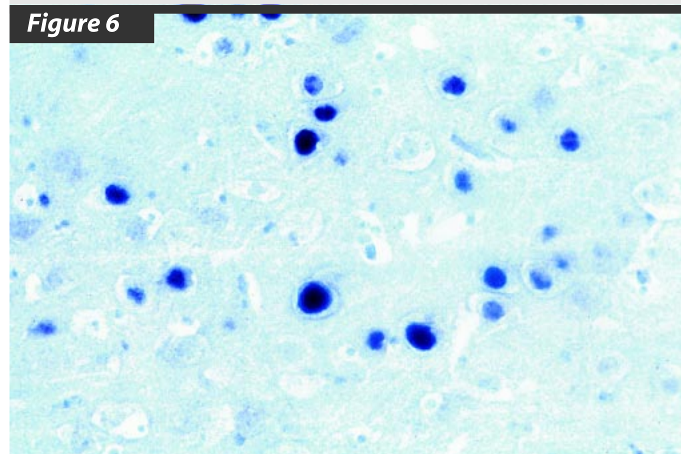
Lung. Nucleic acid of *Pneumocystis carinii* is colored in brown after being detected with a specific probe for *P. carinii* ribosomal RNA. In situ hybridization. Streptavidin-biotin-peroxidase method. Hematoxylin counterstain.

Figure 5



Lung. A closer view of Figure 4. In situ hybridization. Streptavidin-biotin-peroxidase method. Hematoxylin counterstain.

Figure 6



Brain. The blue dots indicate the presence of specific DNA for porcine herpesvirus 1 (pseudorabies). Anti-digoxigenin-alkaline phosphatase method. Fast green counterstain.

Denaturation of the nucleic acid

For hybridization to occur, both the target nucleic acid and the oligonucleotide probe have to be single stranded. This is accomplished using special incubators that achieve high incubation temperatures in the presence of ions and formamide. The equipment costs approximately $2000-$3000.

Hybridization

Once both nucleic acid segments (target and probe) are single stranded, complementary nucleotides of both the target and probe nucleic acid will bind.

Detection systems

Since the first successful reports of ISH,[1–2] there have been dramatic improvements in detection systems. At first, ISH was performed using radioactive probes as the detection system. Although radioactive probes are usually more sensitive and still in use, nonradioactive probes are common today. These nonradioactive probes minimize

health hazards and circumvent governmental laws that regulate work with radionucleides. Diseases for which ISH is being used include transmissible gastroenteritis,[10] pseudorabies,[11] porcine circovirus,[12,13] porcine reproductive and respiratory syndrome virus,[14,15] porcine respiratory coronavirus,[12] foot and mouth disease virus,[16] swine vesicular disease virus,[17] proliferative enteritis,[18] and pneumocystosis.[19]

Interpretation of results

The presence of hybrids indicates a positive reaction (Figures 2–6). If an RNA probe has been used it detects transcription of nuclear material specific for a virus/bacteria; if a DNA has been used it detects viral/bacterial DNA. The number of diseases to which the ISH method has been applied is currently fewer than that for IHC. As for IHC, the significance of a positive result (hybridization between the probe and a segment of nucleic acid on the tissue section) should be assessed in conjunction with clinicopathologic data.

Comparing ISH to IHC for diagnostic purposes

The goals of both IHC and ISH are the same: to demonstrate the presence of material that is specific for an infectious organism in animal tissues. The conceptual approach of these two methods, however, is completely different. One method will be more suitable than the other under varying circumstances. In active infections, where the infectious organism is currently being produced, IHC is preferable because it will detect specific proteins of the actual organism. However, latent infections (such as with herpesvirus) do not produce virus particles and only genomic material from the virus is present in the infected cell, making ISH more suitable than IHC for diagnostic investigation of latent infections. In studies of *Pneumocystis carinii* in porcine lung, we could not detect this microorganism by immunocytochemical methods; with ISH, a strong reaction was observed.[19] The lack of reaction with IHC may have been the result of an inappropriate antisera (not specific for, or not recognizing epitopes of, the porcine *P. carinii*) rather than inadequate technique or the absence of specific antigens. In some cases there is no clear advantage to a particular technique and other factors such as availability of reagents (antisera, nucleic acid probes) are more critical.

Both IHC and ISH are especially useful in retrospective studies because formalin-fixed and paraffin-embedded tissues maintain their structural characteristics for at least several decades. Both techniques are highly specific and sensitive. These methods can distinguish not only genera of organisms but also subtypes and strains, and therefore may be useful in epidemiologic studies. Another advantage of IHC/ISH methods is that they are relatively quick and inexpensive as opposed to some currently used microbiological methods in diagnostic laboratories. In addition, these methods can be automated, increasing the productivity, reliability, and efficiency of the laboratory. The major pitfall with ISH involves a distinction between specific and background staining, because some probe constructions will bind nonspecifically to tissue components such as neurons, glandular epithelium, and collagen.[9] Knowledge of histology and expected staining pattern is essential.

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