Chapter 18

Detecting Decay Fungi with Antibody-Based Tests and Immunoassays

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Early detection of wood decay can prolong the service life of wood. Antibodies are the ideal probe for detecting fungi that cause biodeterioration because they are highly specific and can quantitatively determine the fungal antigen concentration from highly complex structures, such as wood. Polyclonal antibodies recognize multiple chemical sites of the targeted molecule, in our case, a fungal glycoprotein, while monoclonal antibodies recognize one specific protein sequence on the targeted molecule. Both polyclonal and monoclonal antibodies have been utilized separately or in concert to design various assay formats to detect incipient wood decay; depending on the target organism and the test format, an assay can be designed to be specific or broad spectrum, quantitative or qualitative. Immunodiagnostic tests for wood decay fungi include, in order of least to most sensitive, the particle agglutination assay, dot-blot immunoassay, enzyme linked immunosorbent assays (ELISA), and a patented particle capture immunoassay.

Introduction

Wood decay caused by brown-rot decay fungi has typically been described as having four stages, though the stages overlap and cannot be clearly separated *(1)*. Incipient decay occurs when decay fungi initiate colonization and release enzymes; there is no visible evidence of damage. During early decay, slight changes in color or texture occur, but decay is not obvious. Intermediate decay includes obvious changes to color and texture, although the wood structure still appears to be intact. Advanced decay is obvious because the wood structure is affected; the wood turns brown, and crumbly with a cubicle appearance. Sensitive methods for detecting decay in wood, particularly in the initial stages, have long been sought by those responsible for inspecting and maintaining wood in-service *(2)*. Traditional field methods such as sounding of wood, visual inspection of borings, and mechanical probing are useful for detecting advanced decay *(1)*. However, early stages of decay are difficult to detect and incipient decay cannot be detected by traditional field methods *(2,3)*. Traditional laboratory methods such as culturing and microscopic examination are reliable. However, they are time consuming and require professionally trained personnel and laboratory facilities. If a field method can detect decay in a structure before strength reduction occurs, then remedial treatments can be utilized to arrest decay and prevent further damage to wood in-service *(2,4)*.

Recognition of early decay is important for the inspection of wood in service. Early decay is difficult to recognize because it is visually subtle and often occurs on the interior of a timber or below ground. Visually, macroscopic changes in color or texture are presumptive evidence of early decay; only the physical presence of fungi is considered definitive. Microscopic examination revealing hyphae with clamp connections is definitive evidence of early decay *(1)*. Nonvisual methods of identifying early decay include culturing the fungus from infected wood, and various physical tests, such as changes in strength, acoustic, and electrical properties *(3,4)*.

The ideal detection system for diagnosis of fungal decay would incorporate several criteria *(5):*

- Simplicity-easy to use and understand.
- Rapid analysis and accurate results.
- Inexpensive-enables multiple sampling which more accurately diagnoses decay.
- Specific for decay fungi without cross-reaction to non-decay wood-inhabiting microorganisms.
- Small sample size and minimally invasive sampling procedure.

- Portable for use in the field.
- Automated-automation is often mutually exclusive with field testing.
- Quantitative versus qualitative

Selection of antigen and type of antibody are critical elements in the successful development of an immunoassay *(4)*. Ideally, immunizing antigens should originate from the natural substrate, i.e., decayed wood, since there are demonstrated immunogenic differences between fungal enzymes derived from liquid culture versus decayed wood *(6)*. Once a target molecule is identified and antibodies are prepared, determining the antigen extraction method for test samples and optimal antigen concentration are equally critical to the success of an immunoassay. Likewise, choice of assay method and test substrate will influence how to proceed in method design.

Immunological probes

Fungal proteins and polysaccharides elicit an immune response when introduced into a higher animal, typically a rabbit, resulting in antibody production *(7)*. Because antibodies have the capacity to specifically recognize and bind to fungal antigen in a highly complex structure such as wood they are an ideal probe for the detection of fungi that cause biodeterioration. Two distinctly different types of antibodies, monoclonal (Mab) and polyclonal (Pab), have been produced *(8,9)*. Polyclonal antibodies recognize multiple chemical sites, i.e. epitopes, on the antigenic molecule and are typically produced in vivo. Monoclonal antibodies recognize one specific epitope, often a protein sequence on an antigenic molecule *(10)* and are typically produced in vitro by cell culture. When used either separately or in concert, Mab's and Pab's have been utilized to design various immunological tests to detect fungal antigens extracted from wood during the incipient stage of decay *(4,8,10,11)*.

For all methods described in this article, samples consist of wood shavings extracted in an aqueous solution of Triton X-100 (Sigma, St. Louis, MO), which solubilizes the hyphal membrane of the fungus and releases antigens recognized by the antibodies (i.e. Xylanase). Fungal xylanases, which are glycoproteins, vary in the ratio of protein:carbohydrate from fungus to fungus. In laboratory tests, the antibodies used in the following methods recognized brown-rot and white-rot fungi. A broad-spectrum test is desirable for most field inspection applications.

Test Methods

Particle agglutination test (PAT)

Particle agglutination assays are rapid immunodiagnostic tests *(4,8,12)* that have been utilized in the medical field since 1956. Particulate antigens may be agglutinated directly by polyclonal antibodies to surface antigens. Similarly, submicron-sized polystyrene particles coated with polyclonal antibody (Pab) will visibly agglutinate in 30-60 seconds when an antigen is present. Experience is necessary to evaluate reactions to avoid misinterpretation of weak positive reactions. For example, an extracted sample of wood is mixed with antibody-labeled particles in the well of a glass slide and the reaction viewed through the slide with the aid of a light box. A negative result shows a smooth homologous appearance, while a positive test exhibits grainy aggregates of reagent (Figure 1). The strength of agglutination is ranked as negative or 1 to 4, with 4 being the strongest reaction. Particle agglutination has successfully detected infection by brown-rot fungi prior to weight loss, with minimal cross-reactivity to white-rot, mold or sapstain fungi *(8).* This method for decay detection is simple, rapid, specific, and fairly portable, but it is not currently quantitative nor can it be automated.

Quantitative results could, however, be obtained by reading the assay turbidimetrically. Variations are described by Bangs (12) , which both simplify the test portability and increase the reaction complexity. In one variation, antibody-coated particles are dried on a cardboard test strip, and rehydrated with the test sample. Having only one liquid reagent eases field portability. To increase test complexity, multiple antibodies to different fungi or fungal components, can be used to coat polystyrene particles of differing colors. The mixture can then be used either in solution or dried to test samples. Depending on which fungus or component is present in the aqueous test sample, only the corresponding color of antibody-coated bead will agglutinate. Another way to improve portability is to place dry antibody coated particles in the well of a closed-channeled cassette with a zigzag capillary channel. Liquid test sample is added to the particle-containing well and capillary action pulls the reagents through the channel, mixing the antibody-coated particles and test sample as it moves. Agglutination will cloud the cassette indicating a positive reaction and the cassette will remain clear in a negative reaction.

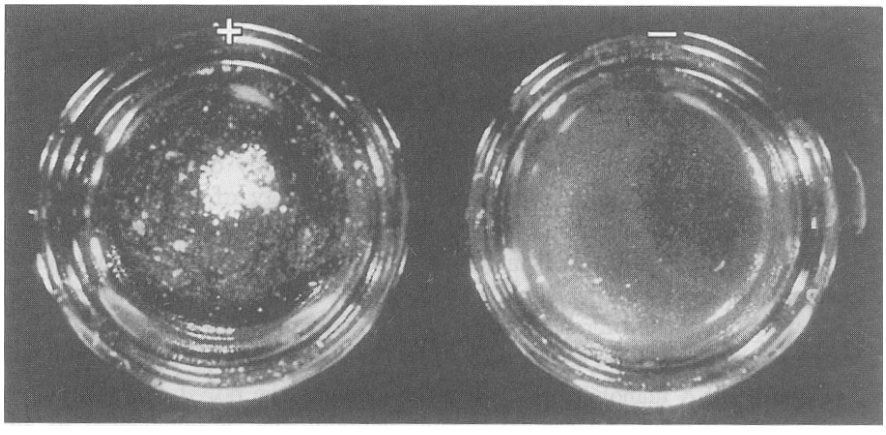

Figure 1. Particle agglutination test (PAT). Left: visible grainy aggregates indicate a positive reaction, right: smooth homogenous particles indicate a negative reaction.

Dot Blot Immunoassay

Blotting membranes offer high protein binding capacity and sensitivity for qualitative detection of decay fungi. Aqueous wood extracts bound to nitrocellulose paper are probed with enzyme-labeled antibody, and addition of substrate yields a visible reaction (Figure 2). A detailed method is described by Clausen and Green *(7).* Glancy *et al. (13)* utilized polyclonal antibodies to a whole cell antigen preparation of *Lepideus lentinus* and Clausen *et al. (8)* utilized a multivalent polyclonal antibody to six common brown-rot fungi to show that simple dot-blot immunoassays are useful for detecting incipient decay *(8).* This method does not lend itself to field portability, nor is it simple, rapid or automated. Dot blots can, however, be analyzed quantitatively by densitometry.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA's give quantitative measurements of antibody or antigen and are performed in 96-well polystyrene plates with a variety of configurations of antigen, antibody and detection systems. While this method cannot be adapted as a field test, it has the advantage of providing automated, quantitative measurements and multiple tests in a single plate. There are several formats of ELISA *(4,8),* including direct, indirect, and double antibody sandwich (DAS). These three methods differ as follows:

- Direct ELISA immobilizes a specific antibody on the polystyrene plate and incubates antigen and second specific antibody that is conjugated to an enzyme or other probe.
- Indirect ELISA immobilizes either antibody or antigen to the polystyrene plate and the enzyme-linked antibody is linked to the immobilized antigen or antibody.
- DAS-ELISA immobilizes a specific antibody to the polystyrene plate and incubates with antigen followed by a second specific antibody. The Ab-Ag-Ab sandwich is incubated with a secondary antibody conjugated with an enzyme.

In all types of ELISA, the last step is to add a chromogenic substrate which will form a visible color reaction when acted on by the enzyme. The reaction can be quantified spectrophotometrically. Alternate methods may use fluorescent or radioactive probes.

Of the above, indirect ELISA has been used most often to detect brown-rot decay (Figure 3). Breuil *(14)* and Breuil *et al. (15)* used ELISA to detect the sapstain fungus, *Ophiostoma* sp. C28 with a polyclonal antibody. Clausen et al.

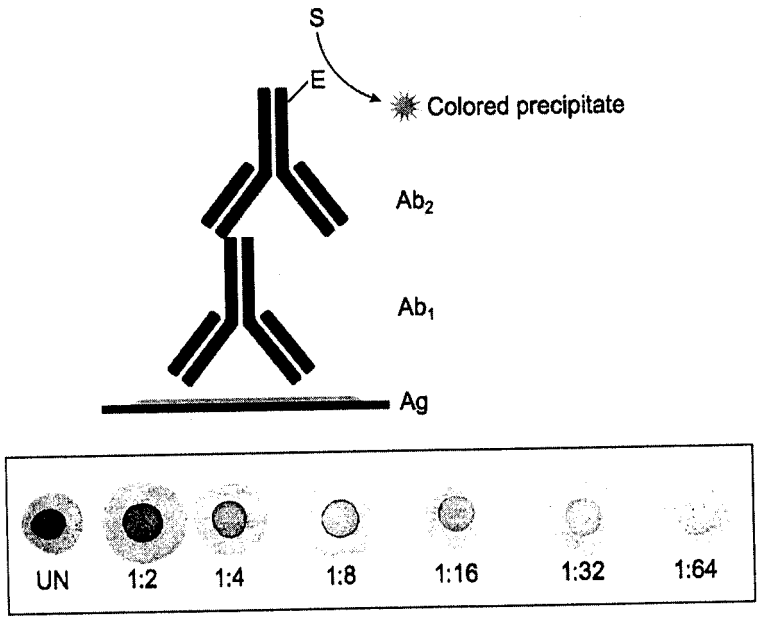

Figure 2. Dot blot immunoassay. Antigen, bound to nitrocellulose membrane, is probed sequentially with antibody(Ab_1) and enzyme-labeled antibody (Ab_2). Addition of substrate yields a colored precipitate. The amount of color is directly proportional to the amount of antigen in the test sample.

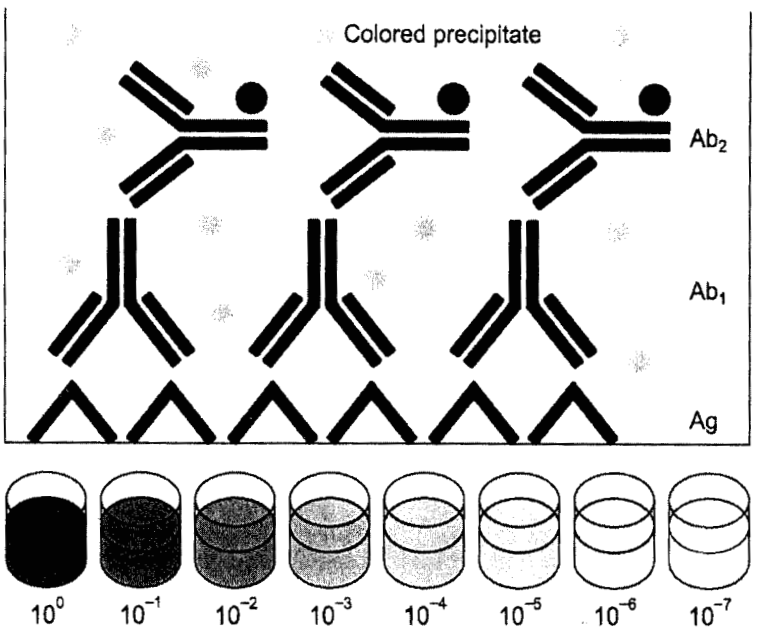

Figure 3. Indirect enzyme-linked immunosorbent assay (ELISA). Top: Schematic ofreaction within each well-antigen, bound to polystyrene wells, is probed with antibody (Ab_1) and enyme-labeled (Ab_2). Addition ofsubstrate yields a coloredprecipitate. Bottom: The amount ofcolor in each well is directly proportional to the amount ofantigen in the test sample.

(5,8) reported detection of six common brown-rot fungi from infected wood at 0-13% wood weight loss using either polyclonal or monoclonal antibodies prepared against Xylanase from *P. placenta.* Goodell and Jellison *(16)* and Goodell *et al. (17)* detected *P. placenta* using polyclonal antibody in samples exposed to the fungus in a soil-block test after 10 days of incubation. A detailed ELISA method can be found in Clausen and Green *(7).*

Chromatographic Immunoassay

Numerous formats exist for chromatographic immunoassays *(12).* All formats rely on either an enzyme-substrate color reaction, such as that used for dot-blots and ELISA's or, more commonly, colored polystyrene particles coated with antibody, like those described in particle agglutination assays. The particle capture immunoassay combines the speed of a PAT with the sensitivity of a double antibody sandwich ELISA. Clausen *(10,11)* and Clausen and Green *(19)* developed and patented a dyed particle capture immunoassay that is superior in sensitivity and specificity to the indirect ELISA. Dyed particles eliminate the need for an enzyme-substrate reaction. In this test, antibody-coated particles move through a porous membrane when an aqueous wood extract is added to a sample well. If antigen is present in the test sample and binds to the antibody-coated particles, it becomes sandwiched by a second anti-xylanase antibody that is immobilized in another location on the porous membrane. The dyed particles also become trapped in the sandwich so that a positive reaction is visualized as a colored line (Figure 4). Unbound particles migrate to a negative control zone. This qualitative test is specific for decay fungi, sensitive to nanograms of xylanase and is able to detect decay in the field prior to weight or strength loss. Alternatively, utilizing an enzyme-substrate color reaction and portable spectrographic device could conceivably result in a quantitative version of the chromatographic immunoassay.

The particle capture immunoassay does not distinguish between active and inactive fungi. Decay fungi can remain inactive in wood for long periods of time when conditions are not favorable for growth; adding sufficient moisture can reactivate fungal growth and decay. By indicating the presence of both active and inactive decay fungi, the particle capture immunoassay can be particularly useful for the inspection of wood in-service, since it alerts the inspector to potential losses if steps are not taken to prevent exposure of the wood to moisture, enables the extent of colonization to be determined, and indicates and defines areas where remedial treatment or replacement are appropriate. Commercial lumber suppliers and mills could monitor the quality of their timber

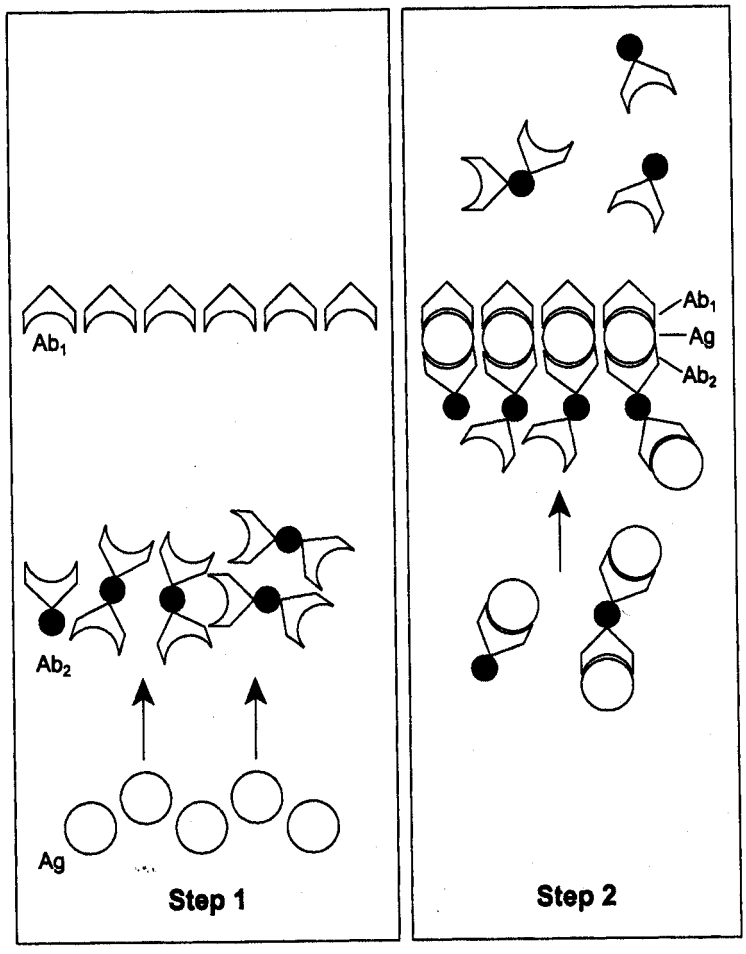

Figure 4. Diagram of the particle capture immunoassay. 1) antigen (liquid wood extract) is applied and migrates through the prepared test strip, 2) antigen captured by Ab_2 coated-particles, migrates to the immobilized antibody (Ab_1) and is sandwiched between Ab_1 and Ab_2, resulting in a colored line, i.e. positive reaction(4).

supply with this method. Since preservatives do not interfer with the antibody/antigen reaction, this assay is also suitable for monitoring treated wood in-service.

Summary

The particle agglutination test, dot blot, ELISA and chromatographic immunoassays are broad immunoassay categories having numerous Variations within each category. Immunoassays have been developed within each category for the detection of incipient fungal decay from aqueous wood extracts. Each method meets most of the criteria for an ideal detection system, and the particle capture immunoassay is conducive to detecting incipient wood decay in the field. The field of immunodiagnostics has made advances through the development of sensitive immunoassays towards a practical field test to detect incipient wood decay.

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