Techniques

An Improved Method for Detection of *Acremonium coenophialum* in Tall Fescue Plants

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ABSTRACT

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The tall fescue endophyte *Acremonium coenophialum* causes a physiological disorder of cattle known as fescue toxicosis. Protein-A sandwich, enzyme-linked immunosorbent assay (PAS-ELISA) was compared to a microscopic staining test and double-antibody sandwich ELISA (DAS-ELISA) and found to be more reliable in predicting infection levels in fescue pastures. Antisera produced against the fescue endophyte cross reacted only to other closely related fungi. The two pastures sampled had infection levels of 57 ± 7 and 80 ± 5%. The PAS-ELISA was superior to DAS-ELISA in that it had lower background absorbance readings from uninfected fescue samples and consistently higher absorbance readings from infected samples. The PAS-ELISA also used less antiserum per sample and agreed more times with the microscopic staining method than did DAS-ELISA. The PAS-ELISA was superior to the microscopic staining method in that more samples could be processed and it was more reliable because the microscopic staining method did not detect hyphae in known infected plants 4% of the time. This is the first report of the use of PAS-ELISA to detect a fungus.

Tall fescue (*Festuca arundinacea* Schreber) is one of the most widely grown forage grass species in the United States, occupying more than 14.5 million ha (5). Cattle that feed on tall fescue infected with the endophytic fungus *Acremonium coenophialum* Morgan-Jones & Gams develop symptoms referred to as "fescue toxicosis." Symptoms in cattle include a reduction in average daily gain, reduced conception rate, elevated body temperature, rough hair coat, and lower lactation. Symptom severity is partially dependent on the percentage of endophyte-infected fescue plants in a given pasture (5). The fungus is disseminated in nature only by seed from infected plants. The endophyte was probably present in the original selection of Kentucky 31 fescue and is currently in most regions where tall fescue is grown (13).

Incidence of the endophyte in fescue plants is determined either

by a staining method (2) or through a direct, double-antibody sandwich, enzyme-linked immunosorbant assay (DAS-ELISA) (6–9,11,12,15). The staining method is limited in that the number of samples that can be processed is less than the number that can be processed with the ELISA techniques. Problems incurred with DAS-ELISA include low absorbance readings for infected fescue and high background absorbance readings for uninfected fescue (15).

The purpose of this study was to compare the mycelium staining method, DAS-ELISA, and an indirect protein-A sandwich ELISA (PAS-ELISA) for accuracy in detecting the fescue endophyte in pasture samples.

MATERIALS AND METHODS

Fungal cultures and fescue samples. *A. coenophialum* was isolated from fescue seed by the method of Conger and McDaniel (3). Fungal cultures of *A. loliae* Latch, Christensen & Sammuels and *Epichloe typhina* (Pers.) Tul. were provided by M. R. Siegel. Cultures were maintained on M43 agar and in M43 broth culture (1). *Drechslera* sp., *Cladosporium* sp., *Penicillium* sp., *Cephalosporium* sp., two *Alternaria* spp., *Fusarium* sp., and *Aspergillus* sp. were maintained at the University of Tennessee and were grown on potato-dextrose agar. Known infected and uninfected tall fescue plants were maintained in isolated clumps under field conditions and were used as positive and negative controls for all tests. Tall fescue samples were collected from two pastures that had been in tall fescue production for 11 and 18 yr. Samples used in the staining test were assigned a number that corresponded to individual stems used for both DAS-ELISA and PAS-ELISA tests.

Antiserum production. Antigens from *A. coenophialum* used to produce antiserum in rabbits were made as follows. Mycelial mats of 10-wk-old cultures of *A. coenophialum* were washed with 4 L of phosphate-buffered saline —0.02 M phosphate, 0.15 M NaCl, and 3 mM KCl, pH 7.3 (PBS)—in a Büchner funnel. Mycelia were suspended in PBS and macerated in a glass tissue homogenizer. The homegenate was centrifuged 10 min at 3,000 *g*. The mycelial culture supernatant was precipitated with 10% (w/v) polyethylene glycol (MW 8,000) and centrifuged 10 min as a modification of the method of Musgrove et al (12). The resulting pellet was resuspended in PBS and injected into a rabbit. Subcutaneous and intramuscular injections of 1 ml of antigen and 1 ml of Freund's incomplete adjuvant were made at weekly intervals for three weeks, followed by one booster injection two weeks later. The rabbit was bled at weekly intervals starting one week after the booster injection.

Microscopic staining method. Tall fescue stems were cut 5–6 cm above the crown of the plant and split longitudinally. The epidermis of an interior leaf sheath was removed and stained with 0.06% aniline blue in 33% lactic acid solution on a microscope slide. The tissue was then observed at 400 × (1).

ELISA. DAS-ELISA was performed according to the methods of McLaughlin et al (10). Three wells were used for each tall fescue sample.

The PAS-ELISA method was devised by Edwards and Cooper (4). Medium binding polystyrene ELISA plates (Plastic Injectors, Spartanburg, SC) were rinsed with water. Protein A (1 μg/ml) (Sigma Chemical Co., St. Louis, MO) in 0.05 M sodium carbonate buffer (pH 9.6) was added to the ELISA plate and incubated at 30 C for 2 hr. Unbound protein was removed by four 30-sec rinses with PBS + 0.05% Tween 20 (PBS-Tween) following each step. Mycelial supernatant antiserum was added at a 1:1,000 dilution in PBS-Tween to each well and incubated 2 hr at 30 C. Fescue stems were pulverized in a leaf squeezer with PBS-Tween (1:5, w/v), and sap from each stem was placed in three wells overnight at 5 C. Antiserum was diluted 1:1,000 in PBS and added to the wells and incubated 2 hr at 30 C. Protein-A alkaline phosphatase was diluted 1:1,000 in PBS-Tween and incubated 2 hr at 30 C. Substrate (p-nitrophenylphosphate, 1 mg/ml) was added to the wells at room temperature, and absorbance ($A_{405\ nm}$) was recorded after 1–2.5 hr.

Known positive (infected) and negative (uninfected) fescue samples were included as controls in each ELISA plate. Wells containing PBS-Tween instead of fescue sap were used to calibrate the Dynatech Minireader II (Dynatech Laboratories, Inc., Alexandria, VA). The positive-negative threshold for PAS-ELISA and DAS-ELISA was set as the mean of the healthy controls plus three standard deviation units (14). Two hundred seventy-five random fescue samples were tested with both the microscopic staining test and PAS-ELISA; 135 of these also were tested with DAS-ELISA.

Tests were conducted to optimize the dilutions of the protein-A step and the two antisera steps used in PAS-ELISA and the two immunoglobulin G (IgG) steps in DAS-ELISA. Tests also were conducted with 11 different fungal isolates to observe cross reactivity of the antiserum.

RESULTS AND DISCUSSION

With the microscopic staining test, *A. coenophialum* hyphae were easily recognized in fescue tissue because of their serpentine appearance. In three samples, straight, septate hyphae were observed; all three samples were negative for the endophyte in the PAS-ELISA tests.

Optimized conditions for DAS-ELISA were a 1:200 (5.75 μg) dilution for both the coating and detecting antibodies. Samples tested positively up to a 1:800 dilution of either IgG step, and as little as 10 μg of homogenized *Acremonium* could be detected. An antiserum dilution of 1:1000, the equivalent of 2.3 μg of IgG, was the optimum for both the first or second antibody steps for PAS-ELISA. However, for PAS-ELISA, samples tested positively with as great as 1:128,000 dilution of the first antibody and a 1:8,000 dilution of the second antibody, and as little as 0.01 μg/ml of protein A. The sap dilution end point for infected fescue tissue was 1:1,280. As little as 40 ng of homogenized mycelium could be detected with PAS-ELISA.

Other fungal mycelia were tested with PAS-ELISA for possible cross-reactivity. Mycelia (1 mg/ml) of *Drechslera* sp., *Cladosporium* sp., *Penicillium* sp., *Cephalosporium* sp., two *Alternaria* spp., *Fusarium* sp., and *Aspergillus* sp. were homogenized in PBS containing an extract of healthy fescue (1 g of fescue in 12 ml of PBS) and tested negatively with PAS-ELISA. Two *A. loliae* and one *E. typhina* isolates reacted to the *A. coenophialum* antiserum but with lower absorbance readings than the homologous reaction. The same results were observed using DAS-ELISA, except that all unrelated isolates had a slightly higher absorbance reading than occurred with healthy fescue sap alone. These results agree with those of Musgrave et al (12) and Johnson et al (7) who also found little reactivity between their antisera and other unrelated fungi.

The infection levels of the two pastures used in the test averaged 57 ± 7 and 80 ± 5% for four and six different test dates, respectively, with approximately 20 samples per date.

The mean absorbance values for negative controls of DAS-ELISA and PAS-ELISA were 0.05 ± 0.08 and 0.04 ± 0.04, respectively. The mean ELISA indices ([sample mean − negative control mean]/ standard deviation of negative control mean) for infected DAS-ELISA were 8.1 ± 5.1 and for PAS-ELISA were 15.7 ± 7.22 (Table 1). The mean PAS-ELISA index for most uninfected fescue samples was less than that of the healthy control samples used to calculate the ELISA indices (note that a value greater than 3 would be a positive sample). However, the mean DAS-ELISA index for most uninfected pasture samples is one standard deviation above that of the negative controls (Table 1). Thus, test results are easier to interpret using PAS-ELISA because of the larger difference in the ELISA indices between infected and uninfected samples (14).

Agreement between the microscopic staining method and PAS-ELISA results was 95%. *Acremonium*-like hyphae were observed in only 1 of 275 fescue samples in which the PAS-ELISA reading was negative. Therefore, the chances of detecting a false negative sample in this study using PAS-ELISA was less than 1% (Table 2). Hyphae were not observed in 13 of 275 (5%) fescue samples in which the PAS-ELISA readings were positive (Table 2). There are

TABLE 1. Comparison of enzyme-linked immunosorbent assay (ELISA) indices using double-antibody sandwich (DAS)-ELISA and Protein-A sandwich (PAS)-ELISA to detect *Acremonium coenophialum* in tall fescue plants

Method	ELISA index[a]	
	Infected samples	Uninfected samples[b]
PAS-ELISA	15.66[c] ± 7.22[d]	−0.20 ± 0.89
DAS-ELISA	8.11 ± 5.13	1.07 ± 1.14

[a] ELISA index is (fescue sample mean absorbance − healthy control mean absorbance)/standard deviation of the healthy control mean.
[b] The healthy control mean absorbance plus 3 standard deviations was used as the positive-negative threshold; therefore an ELISA index greater than 3 would be an infected sample.
[c] Mean.
[d] Standard deviation.

TABLE 2. Comparison of the microscopic staining method (MSM), Protein-A sandwich (PAS) enzyme-linked immunosorbent assay (ELISA) and double-antibody sandwich (DAS)-ELISA for detecting *Acremonium coenophialum* in tall fescue plants

Reaction of test method[a]				
MSM	PAS-ELISA	DAS-ELISA	Samples (no.)	Total (%)
+	+		168	61
−	−		93	34
+	−		1	0.4
−	+		13	5
+		+	91	67
−		−	25	18
+		−	3	2
−		+	16	12
	+	+	95	70
	−	−	26	19
	−	+	2	1
	+	−	12	9

[a] Positive (+) = infected; negative (−) = uninfected.

several possible explanations for this discrepancy: 1) PAS-ELISA gave false positive readings; 2) hyphae were present but were not observed with the microscopic staining method; 3) hyphae were present in the fescue plant but not in the tiller tested and thus PAS-ELISA was reacting to some transportable antigen; or 4) a combination of two or more of the above. To test which of these explanations was plausible, 50 tillers from known infected plants were tested with PAS-ELISA and the microscopic staining method. All were positive or infected according to PAS-ELISA tests, but no hyphae were observed in 4% of these samples. Thus, PAS-ELISA appeared to be more reliable (99%) at detecting infected fescue plants than the microscopic staining method.

The DAS-ELISA method was compared with the microscopic staining method and PAS-ELISA methods (Table 2); agreement was found on 86 and 90% of the samples, respectively. When the microscopic staining method or PAS-ELISA was positive, DAS-ELISA disagreed 2 and 1% of the time, respectively. When the microscopic staining method or PAS-ELISA was negative, DAS-ELISA was positive for 12 and 9% of the samples, respectively (Table 2). Thus, if, as above, 4% of the time hyphae were not observed in infected plants using the microscopic staining method, then the DAS-ELISA is incorrectly predicting infected plants 8–9% of the time when compared with both the microscopic staining method and PAS-ELISA. This error is most likely due to the erratically high background readings of some negative control samples when using DAS-ELISA.

This is the first report of the use of PAS-ELISA to determine fungal infection levels. No test to date is 100% accurate; however PAS-ELISA is an improvement over DAS-ELISA. PAS-ELISA has the following advantages: It has no antibody isolation or conjugation step; less antibody is used per sample; and PAS-ELISA is more accurate. Both ELISA methods have the disadvantage of requiring animal facilities for antiserum production and specialized equipment, such as the ELISA reader, which may not be available in all laboratories. PAS-ELISA appears to be more reliable at determining infection levels than the microscopic staining method. This may be because PAS-ELISA may be more sensitive and because a larger number of samples can be processed with either ELISA method than with the microscopic staining method. Until a test for the toxic element(s) produced by the endophyte is found, PAS-ELISA will be useful in predicting endophyte levels in pastures so that informed decisions concerning pasture renovation can be made by livestock producers.

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