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Immunoblot Analysis of the Humoral Immune Response to *Pythium insidiosum* in Horses with Pythiosis

LEONEL MENDOZA,†* VIVIAN NICHOLSON, AND JOHN F. PRESCOTT

Department of Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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Reactions to *Pythium insidiosum* by sera from horses with active pythiosis were investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Five strains of *P. insidiosum* were grown in nutrient broth and then sonicated. After centrifugation, supernatant antigens were separated by SDS-PAGE. An exoantigen of *Conidiobolus coronatus* was also tested. Bands with molecular weights between 97,000 and 14,000 were identified by Coomassie blue and silver staining. After being transferred to nitrocellulose, the antigens were reacted against sera from six horses with pythiosis, sera from four horses cured a year earlier by vaccination, and sera from five healthy horses. The sera from horses with pythiosis recognized at least 20 antigens in all strains. Three antigens with molecular weights of 32,000, 30,000, and 28,000 appeared to be immunodominant and specific. Sera from horses cured by immunotherapy showed only five very weak bands, three of them the 32,000-molecular-weight (32K), 30K, and 28K antigens. No bands were observed with sera from healthy horses or sera from horses with a variety of other infections. Sera from horses with pythiosis cross-reacted with the 44K antigen of *C. coronatus*. The immunodominant antigens described here may be useful for diagnostic purposes and in immunotherapy for this oomycotic infection in horses.

Pythiosis is a subcutaneous, bone, or lung disease of horses (1, 7, 12), cats (3), and cattle (18); an intestinal and/or subcutaneous disease of dogs and horses (5, 19, 20); and a cutaneous, subcutaneous, and blood vessel disease of humans (22, 23, 25). The etiological agent was long known as *Hyphomyces destruens*, an invalid name based on the epithet given to this disease in 1902 (4). A strain isolated from a horse in New Guinea was classified as a *Pythium* sp. (2) on the basis of its ability to produce biflagellate zoospores; similar strains were later recovered from horses in other tropical and subtropical countries (6). On the basis of these findings, some authors speculated that several *Pythium* spp. were involved as agents of this disease in animals (19). However, De Cock et al. (6), working with strains isolated from animals and humans from different geographical locations, determined that a single species, *Pythium insidiosum*, was the etiological agent of this oomycotic infection, a finding later corroborated by others (13, 14).

Horses in the early stages of this disease react positively in a delayed hypersensitivity skin test using a concentrated culture filtrate antigen (CFA) (11) and are cured by vaccination with antigens acquired through precipitation with acetone (15). These reports and the fact that sera from the majority of horses with active pythiosis reacted positively in the immunodiffusion (ID) test (12, 15, 17) suggest that the cellular and humoral immune responses are active early in infection. Animals with pythiosis become anergic after the disease reaches the chronic stage (2 months and later) (11, 15, 16).

Little information on the nature of the antigens eliciting antibodies in horses afflicted with pythiosis is available. Miller and Campbell (17) and Mendoza and Alfaro (11) developed an ID test that detected one precipitogen from trypsin-disrupted hyphae of *P. insidiosum*. A less complex and more stable antigen for the ID test was developed later (12). The latter CFA detected three to six precipitins in sera from *P. insidiosum*-infected horses and humans. One of them was the antigen reported earlier. The CFA proved to be useful not only for diagnosing pythiosis in cattle (6), cats (3), dogs (12), horses (1, 12, 15), and humans (8, 21–23) but also for monitoring the response to treatment (12, 21).

Besides the six precipitogens reported by the ID test, there are no data on the antigens of *P. insidiosum* that are important during infection. The aim of this study was to investigate further the antigens of *P. insidiosum* which may be important in the immune response of horses to *P. insidiosum* infection.

MATERIALS AND METHODS

Strains. Antigens from five strains of *P. insidiosum* and one strain of *Conidiobolus coronatus* were prepared. The following isolates of *Pythium insidiosum* were studied: CBS 574.85 = ATCC 58643 = Costa Rica-H9, isolated from horses with pythiosis in Costa Rica (type culture); CDC B-4301 (60932), isolated by R. I. Miller from horses with pythiosis in the United States; ATCC 46947 = K-K-16-77-Japan, isolated by Ichitani and Amemiya from horses with pythiosis in Japan; S-5 = Costa Rica-H19, isolated by A. W. Shipton from horses with pythiosis in Australia; and CBS-623.85, isolated from humans with active pythiosis in Thailand. In addition, *C. coronatus* ATCC 60925 = Costa Rica-C5, isolated from horses with subcutaneous zygomycosis in Costa Rica, was included.

Antigen production. The *P. insidiosum* strains were grown on Sabouraud dextrose agar by subculturing the samples every 20 days at room temperature. The cultures were then transferred to cornmeal agar slants (Difco Laboratories, Detroit, Mich.) 10 days before being inoculated in nutrient

* Corresponding author.
† Present address: Department of Microbiology, University of Texas at Austin, Austin, TX 78712-1095.

broth (Difco). Briefly, small portions of growth from the cornmeal agar slant cultures of all *P. insidiosum* isolates were transferred to 1.0-liter flasks containing 500 ml of nutrient broth (Difco) and then incubated at 37°C on a shaker rotating at 150 rpm for 5 days. After 5 days of growth, the cultures were killed with Merthiolate (0.02% wt/vol), incubated one more day on a shaker at 37°C, and then filtered. The sterility was checked. The cell masses obtained by filtration were washed three times with sterile distilled water and resuspended in 5 ml of distilled water after the last wash. The suspension was transferred to 50-ml beakers on ice and sonicated with a Bronson Sonifier, model 200 (Bronson Sonic Power Co., Danbury, Conn.), until 100% of the mycelial mass was disrupted, as determined by light microscopy. The samples obtained after sonication were centrifuged at 5,000 × *g* for 20 min, and the supernatant was collected and used to provide antigens. The protein concentrations of the samples were estimated by the Bio-Rad microassay procedure (Bio-Rad Laboratories, Mississauga, Ontario, Canada), and samples were stored at 4°C until they were used.

In addition to the *P. insidiosum* sonicated antigens, a CFA of *C. coronatus* was prepared (9). Portions of *C. coronatus* growth from cornmeal agar slants were transferred to 1.0-liter flasks containing 500 ml of brain heart infusion broth, and the cultures were incubated at 37°C on a shaker rotating at 150 rpm. The flasks were removed after 5 days of incubation, and the cultures were killed with Merthiolate (0.02%) and filtered. The filtrate was concentrated by ultrafiltration under positive pressure in a stirred cell fitted with a PM-10 membrane (Amicon Corp., Lexington, Mass.) and stored at 4°C until they were used.

Sera. Five serum samples were collected from Costa Rican horses with pythiosis. The disease was confirmed by isolation of *P. insidiosum* in pure cultures, histopathology, and/or serology (12). For control purposes, the following serum samples were evaluated: three serum samples from horses with proven habronemiasis, serum from a horse with proven sporotrichosis, serum from a horse with a *Streptococcus* sp. infection, three serum samples from horses cured of pythiosis by immunotherapy (1 year after vaccination) (15), and five serum samples from healthy horses. Sera were stored at −70°C until they were used.

SDS-polyacrylamide gel electrophoresis (PAGE). Ten-microliter samples containing up to 20 μg of protein were added to an equal volume of sample buffer consisting of 125 mM Tris-HCl (pH 6.8), 6% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, and 0.05% bromophenol blue and boiled for 10 min. Samples were centrifuged at 14,000 × *g* and subjected to electrophoresis on a 12% (wt/vol) polyacrylamide resolving gel and a 4% (wt/vol) stacking gel as described by Laemmli (10). Gels were stained with either Coomassie brilliant blue or silver stain (Bio-Rad Laboratories) and the molecular weights were determined with M_r standard (Pharmacia LKB, Uppsala, Sweden).

Immunoblotting. Antigens were electrophoretically transferred from one-dimensional SDS-PAGE gels to nitrocellulose membranes (0.2-μm pore size) according to the method described by Towbin et al. (26). After being transferred overnight, the nitrocellulose membranes were blocked with 5% gelatin in phosphate-buffered saline (pH 7.4) containing 10% (wt/vol) Tween 20. Horse sera were diluted 1:500 in the 5% gelatin blocking solution. The membranes were then incubated at 37°C for 1 h in these sera with shaking before being washed in the PBS-Tween solution. Horse antibody binding was detected with alkaline phosphatase-conjugated goat anti-horse immunoglobulin $G_{(H\&L)}$ at a 1:1,000 dilution (vol/vol) in the gelatin blocking solution. The membranes were then washed with PBS-Tween, and the antibody reaction was developed with 4-chloro-1-naphthol as the chromogenic substrate.

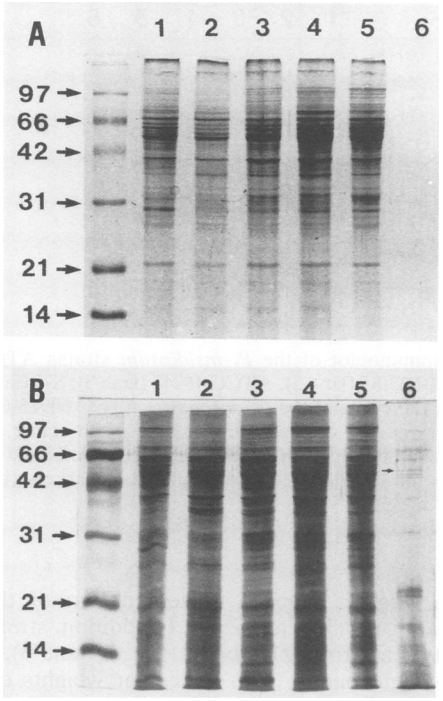

FIG. 1. SDS-PAGE profiles by Coomassie blue (A) and silver (B) staining of the five sonicated *P. insidiosum* strains ATCC 58643 (lane 1), CBS-623.85 (lane 2), ATCC 46947 (lane 3), S-5 (lane 4), and CDC B-4301 (lane 5) and the *C. coronatus* ATCC 60925 CFA (lane 6). The arrow in panel B shows the location of the 44K antigen of *C. coronatus*. Numbers on the left indicate molecular mass markers (in kilodaltons).

RESULTS

SDS-PAGE. All five strains of *P. insidiosum* stained with Coomassie brilliant blue exhibited similar profiles by SDS-PAGE. Many bands ranging from 97,000 to 14,000 and lower in molecular weight were observed (Fig. 1A). At least 33 to 38 bands were detected in each strain. The *C. coronatus* CFA did not show visible bands (Fig. 1A, lane 6). Most bands were located at high molecular weights (97,000 to 28,000) in the five *P. insidiosum* strains. Bands ranging from 100,000 to 14,000 and lower in molecular weight were detected in silver-stained gels (Fig. 1B). An increase in the number of bands in all *P. insidiosum* strains, especially in the lower-molecular-weight range (28,000 and lower) was observed with these gels. The *C. coronatus* CFA (Fig. 1B, lane 6) showed at least 16 weakly staining bands, 9 of them at high molecular weights (97,000 to 31,000) and the remaining bands at 31,000 and lower.

Immunoblotting. The five serum samples from horses with proven pythiosis recognized at least 20 antigens observed in the SDS-PAGE gels (Fig. 2). Most of the antigens detected by these sera ranged in molecular weight from 68,000 to 14,000. The 32,000-molecular-weight (32K), 30K, and 28K bands were particularly prominent. The three immunodominant bands stained with the same intensity (Fig. 2, arrows).

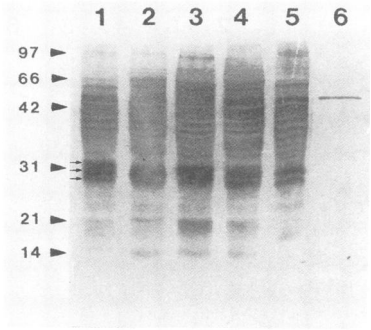

FIG. 2. Immunoblot of the *P. insidiosum* strains ATCC 58643 (lane 1), CBS-623.85 (lane 2), ATCC 46947 (lane 3), S-5 (lane 4), and CDC B-4301 (lane 5) and the *C. coronatus* ATCC 60925 CFA (lane 6) after being reacted with sera from horses with active pythiosis. Arrows shows the 32K, 30K, and 28K immunodominant antigens. Numbers on the left indicate molecular mass markers (in kilodaltons).

One strain (Fig. 2, lane 5) presented two of the three prominent antigens (30K and 28K). In addition, strain ATCC 46947 showed a strong 21K band (Fig. 2, lane 3). Higher-molecular-weight bands, with molecular weights of 68,000 and higher, were weakly detected by the immunoglobulin G in the five horse serum samples tested. Sera from the five horses with pythiosis cross-reacted with the 44K antigen of the CFA of *C. coronatus* (Fig. 2, lane 6). This antigen was part of the three close bands with molecular weights between 44,000 and 43,000 detected in SDS-PAGE by silver staining (Fig. 1B, arrow).

No immunoblot reaction was observed with five serum samples from healthy horses or with any of the other sera from horses with various other diseases. Three serum samples from horses cured by immunotherapy showed five very weak bands (data not shown) after being reacted with the same antigens, three of them the 32K, 30K and 28K antigens. We also observed a hint of the 21K and 20K low-molecular-weight antigens. None of these sera cross-reacted with the *C. coronatus* CFA.

DISCUSSION

Our SDS-PAGE analysis revealed that the five *P. insidiosum* strains studied presented similar antigenic profiles. Gels stained with Coomassie blue showed identical bands for the strains tested, although minor differences were detected, especially with lower-molecular-weight bands (Fig. 1). The *C. coronatus* CFA and the lower-molecular-weight bands of *P. insidiosum* detected in silver-stained gels may be glycoproteins or carbohydrates, since they were either weakly detected or not detected at all by Coomassie blue staining. Some glycoproteins of similar molecular masses have been identified in the CFA of a *Basidiobolus* species (24). Further studies are necessary to investigate the true chemical nature of these silver-staining bands in *P. insidiosum*.

Immunoblotting showed that the immunoglobulin G in the sera of horses with pythiosis recognized most of the bands (68K to 28K bands) detected by Coomassie blue and silver staining (Fig. 2). Immunoblot analysis also indicated that all *P. insidiosum* strains shared the same antigenic profiles. Importantly, the five strains tested were isolated from different geographical locations. In earlier studies, *P. insidiosum* strains from humans or animals shared the same number of bands after being reacted in ID tests with sera from horses with pythiosis (6, 12, 13, 21). Our data confirmed that *P. insidiosum* strains are antigenically similar, regardless of the source. All strains presented similar intensities and numbers of bands in the ID test against the five horse serum samples. However, the numbers and intensities of precipitins observed in the ID test did not show correlation with band intensities on the immunoblot. For example, strain ATCC 46947 showed four immunodominant antigens (Fig. 2, lane 3), while strain CDC B-4301 (Fig. 2, lane 5) showed only two of the four prominent antigens. Six bands were recorded in the ID test when the five horse serum samples were reacted against these two strains.

The 32K, 30K, and 28K immunodominant antigens found after immunoblot analysis were also of major interest. Since reports of cases of pythiosis in humans and animals have increased in the past decade, the recognition of such species-specific antigens, which might play a role in immunity from and diagnosis of this disease, is important. These three antigens may be useful in the development of a more sensitive diagnostic test to delineate the enzootic areas of animal or human pythiosis. Our results also showed that sera from horses cured 1 year previously by immunotherapy reacted weakly with the 32K, 30K, and 28K prominent antigens. By contrast, cases such as these, in which patients were cured, had shown negative results in the ID test (12, 21), but this may be because of this test being less sensitive than immunoblotting. The finding of immunoglobulin G to these three antigens 1 year after the horses were cured suggests that the prominent antigens may also be important as protective immunogens in horses.

It was reported previously that some horses with pythiosis were cured after vaccination with hyphae or the CFA of *P. insidiosum*, but the same vaccines were ineffective in other cases (12, 16). In a recent study, however, Mendoza et al. (15) recommended vaccination in the early stages of infection, on the basis of findings that vaccinated horses responded to treatment according to the age of their lesions. Since no specific antigens have been shown to be associated with the cure of horses infected with this organism, our results open the possibility for the specific use of these three specific antigens as candidates for vaccination trials.

Previous reports have shown that sera from horses with subcutaneous zygomycosis caused by *C. coronatus* did not cross-react with antigens of *P. insidiosum* in the ID test (11), but the antigenic relationship between sera from horses with pythiosis and *C. coronatus* antigens was not clarified. Our data showed that sera from horses with pythiosis cross-reacted with the 44K CFA of *C. coronatus*. This antigen was found to be part of the three bands detected between 44 and 43 kDa (Fig. 1B). Kaufman et al. (9) found two cross-reactive bands when sera from horses with active pythiosis were tested against antigens of *Basidiobolus haptosporus* in an ID test. One of these antigens showed identity with *C. coronatus* when sera from horses with subcutaneous zygomycosis caused by *C. coronatus* reacted against *B. haptosporus* antigens. Yangco et al. (27), using CFA of these zygomycetes and rabbit sera, reported similar findings. Since the band that showed identity with *B. haptosporus* antigens and serum from a horse with *C. coronatus* was the same one that cross-reacted against sera from horses with pythiosis and antigens of *B. haptosporus*, it is possible that the 44K band of *C. coronatus* detected in this study might be the same antigen that showed identity with *B. haptosporus* in the ID test.

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