Journal of Nematology 29(3):268–275. 1997.

Antibodies from Chicken Eggs as Probes for Antigens from *Pasteuria penetrans* Endospores[1]

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Abstract: The bacteria *Pasteuria* spp. have been identified as among the most promising of several microbial organisms currently under investigation as biological control agents of plant-parasitic nematodes. As part of our goal to develop methods to discriminate isolates of *Pasteuria penetrans* with different host preferences, we investigated the potential of developing antibody probes to identify endospores of different isolates of *P. penetrans*. Polyclonal IgY antibodies were raised in chickens against endospores of *P. penetrans* isolates P20 and P100. Hens were injected with P20 or P100 endospore suspensions and boosted at 14 days. Anti-spore titers were determined with ELISA on yolk extracts of individual eggs as a function of time. The highest titers were found in eggs produced at 22 to 35 days after initial injections. Yolk extracts showing the highest titers were combined and processed to provide partially purified IgY preparations. SDS-PAGE and immunoblot analyses identified protein antigens with M_r values of 23–24, 46, and 57–59 KDa common to both P20 and P100 endospores. One protein antigen with an M_r value of 62 KDa was unique to the P100 endospores. The IgY antibodies reduced the attachment of *Pasteuria* endospores to their nematode hosts, indicating antibody interaction with antigens on the endospore surface that are involved in the recognition and attachment processes.

Key words: antibody, antigen, bacterium, biological control, ELISA, endospore, immunoblot, *Meloidogyne arenaria, Meloidogyne incognita, Meloidogyne javanica, Pasteuria penetrans,* protein, root-knot nematode, SDS-PAGE.

Pasteuria spp. comprise a group of endospore-forming bacteria that parasitize nematodes. *Pasteuria penetrans,* the best known member of this genus, parasitizes root-knot nematodes, *Meloidogyne* spp. (Sayre and Starr, 1985). Some reports show that *P. penetrans* suppresses nematode population densities below economic damage levels when endospore densities increase over time (Bird and Brisbane, 1988; Chen et al., 1996; Dickson et al., 1994; Minton and Sayre, 1989; Oostendorp et al., 1991; Weibelzahl-Fulton et al., 1996).

Other *Pasteuria* species include *P. thornei* Starr & Sayre (Starr and Sayre, 1988), which parasitizes *Pratylenchus* spp., and *Pasteuria nishizawae* Sayre, Wergin, Schmidt, & Starr (Sayre et al., 1991), which parasitizes *Heterodera* and *Globodera* spp. In recent taxonomic studies of the bacterium, isolates from *Heterodera goettingiana* (Sturhan et al., 1994), *Belonolaimus longicaudatus* (Giblin-Davis et al., 1995), and *Mesocriconema ornatum* (Hewlett and Dickson, pers. comm.) are considered undescribed species.

The delimitation of *Pasteuria* spp. is based mainly on host ranges and on morphology of endospores. Many observations of *Pasteuria* spp. endospores associated with a wide variety of soil nematodes, including those most important to agriculture, suggest that a large number of species remain to be identified and described (Sayre and Starr, 1988). It is not clear to what extent isolates within a species differ genetically, physiologically, and taxonomically, so new approaches are needed to aid in defining species.

Studies on the attachment of *P. penetrans* endospores to the nematode cuticle show that many isolates vary in their specificity for certain nematode species and populations (Davies et al., 1988; Hewlett and Dickson, 1993; Oostendorp et al., 1990; Sayre and Starr, 1988; Stirling, 1985). The mechanisms involved in the attachment are poorly understood. Proteins may be involved in attachment (Davies et al., 1992; Persidis et al., 1991). A lectin with ligand-binding properties similar to wheat germ agglutinin inhibited attachment of endospores of *P. pen-*

Received for publication 30 December 1996.

[1] Florida Agricultural Experiment Station Journal Series No. R-05440. Supported in part by the Florida Peanut Producers Check Off Funds and by the Interdisciplinary Research Initiative of the Research and Technology Investment Fund, University of Florida.

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etrans to nematodes, implicating a recognition of N-acetyl-D-glucosamine residues on the surface of the host (Bird et al., 1989; Davies and Danks, 1993; Persidis et al., 1991). Polyclonal antibodies raised in rabbits against endospores from the PP1 isolate of *P. penetrans* were used to detect antigens involved in attachment of endospores to nematode cuticles (Davies et al., 1992; Davies and Danks, 1993; Persidis et al., 1991). These antibodies have been used to identify a number of protein bands resolved with SDS-PAGE.

The objective of this work was to evaluate chicken eggs as a rapid and convenient source of antibody probes for antigens specific to the endospores of *P. penetrans*. This study describes the preparation of antibodies (IgY, alias IgG) (Ambrosius and Hadge, 1987, Leslie and Clem, 1969), directed against endospores from *P. penetrans* isolates P20 and P100 and application of these antibodies to detect antigens from endospores of *P. penetrans*.

Materials and Methods

Isolates of Pasteuria: Two isolates of *P. penetrans* were tested—P20 with a host preference for *M. arenaria* race 1, and P100 with a host preference for *Meloidogyne incognita* race 1 and *M. javanica*. Isolate P20 was obtained from *M. arenaria* race 1 from a peanut field in Levy County, Florida, and P100 from *Meloidogyne* sp. from Pasco County, Florida (Oostendorp et al., 1990). Specimens of both isolates are being maintained as greenhouse cultures and stored in dry sand in the laboratory.

Purification of endospores: Second-stage juveniles of *M. arenaria* race 1 and *M. incognita* race 1 were exposed to endospores of *P. penetrans* isolates P20 and P100, respectively (Hewlett and Dickson, 1993). They were then inoculated onto tomato cv. Rutgers seedlings and placed in a greenhouse. After 55 days the plant roots were collected and incubated overnight in Cytolase PCL 5 (Genencor International, Rochester, NY) and washed vigorously on an 800-µm-aperture sieve nested on a 250-µm-aperture sieve (Dickson et al., 1970). The females and plant debris were washed into a counting dish and the endospore-filled females were hand-picked, washed, and then crushed in deionized water. The mixture of female body parts and endospores was washed on an 8-µm-aperture nylon filter; the endospores that passed through were collected and centrifuged (microcentrifuge) for 5 minutes. For analysis of cross-reactive antigens, the pellet containing the endospores was suspended in 1 ml of a saturated solution of sodium diatrizoate. This suspension was injected into the bottom of an 8-ml linear gradient (0.2 M to 0.8 M) of sodium diatrizoate and centrifuged at 15,000g (Beckman JS-13 rotor at 10,000 rpm) for 30 minutes at 20 °C. A single band at a position 60% from the meniscus was removed with a syringe and diluted to more than three times the original suspension volume with deionized water; the endospores were then collected following brief centrifugation in a microfuge. The endospores were washed two times with deionized water by centrifugation, resuspended in deionized water, and counted with a hemocytometer. The endospore suspension was evaluated with an optical microscope to assess purity and properties of the endospores, and stored in a refrigerator at 4 °C. These purified endospores were evaluated in an enzyme-linked immunosorbance assay (ELISA) or were extracted to prepare denatured protein for comparison by SDS-PAGE and immunoblotting.

Polyclonal antibody preparation: The polyclonal antibodies were raised in White Leghorn hens against endospores of *P. penetrans* isolates P20 and P100. Endospores were passed through a 25-µm-aperture sieve before injection. Aqueous endospore suspensions of P20 at 4×10^5 endospores/ml and P100 at 4.5×10^5 endospores/ml were prepared. Each hen was injected with 720 µl of the endospore suspension in the wing (subcutaneous) and 320 µl of the endospore suspension in the footpad. Two hens were used for each *P. penetrans* isolate. A boost injection was given 14 days after the initial injection. The eggs laid by the hens were collected every day following the initial injec-

tion and stored at 4 °C until used. To identify the time for maximum antibody formation, eggs collected every 3 to 5 days were individually extracted and analyzed for titers against immobilized endospores. The intact egg yolks were removed and rinsed with 0.1 M sodium phosphate buffer. The yolk from each egg was lysed in 60 ml of 0.1 M sodium phosphate buffer, pH 7.6, and stirred for 30 minutes. The activity of antibodies in 1/100 dilution of the yolk suspension in PBST (10 mM sodium phosphate buffer, pH 7.6, 0.9% sodium chloride, and 0.2% Tween 20) was determined by enzyme-linked immunosorbance assay (ELISA). Egg yolks containing a high level of antibody activity were collected as a pool, and the IgY fraction was purified following the procedure described by Polson et al. (1985). The activities of the IgY fractions were determined by ELISA.

General procedure of ELISA: Each well of a 96-well microtiter plate was coated with 50 µl of 10 ppm poly-L-lysine (Sigma, St. Louis, MO) in PBS (10 mM sodium phosphate buffer, pH 7.6, 0.9% sodium chloride) for 2 hours at room temperature and washed with PBS four times. The gradient-purified endospores were delivered to those wells designated to receive antigen. To each of the remaining wells, 50 µl of PBS was added. After incubation for 2 hours at room temperature, the plates were washed in PBS four times, fixed with 100 µl of 0.25% glutaraldehyde for 3 minutes, washed with PBS four times, and incubated with blocker (1% bovine serum albumin, 0.02% sodium azide, and 0.1 M glycine) for 2 hours at room temperature or overnight at 4 °C. The plates were then washed with PBST four times. Depending on the titer of the antibody, appropriate dilutions of IgY antibody samples were then added. The plates were incubated at room temperature for 2 hours and washed with PBST four times. To each well, 75 µl of 1/2,000 dilution of an alkaline phosphatase conjugate of rabbit anti-chicken IgG (Sigma) in PBST was added and incubated for 2 hours at room temperature. The plates were washed four times with PBST. To each well, 75 µl of 0.1% *p*-nitrophenyl phosphate di(Tris) salt solution (Sigma) in 0.05 M Na_2CO_3:0.05 M Na_2HCO_3:0.0005 M $MgCl_2$ was added. The reaction was monitored by reading the absorbance at 405 nm on a Model 2550 EIA Reader (BioRad, Hercules, CA).

Determination of activities of IgY in yolk extracts: Excepting the antigen-minus control wells which received PBS, 50 µl of an endospore suspension (isolates P20 or P100) of 2.5×10^5 endospores/ml in PBS was added as antigen. Yolk suspension was centrifuged with a microcentrifuge for 2 minutes before dilution and assay. From each sample, 100 µl of supernatant of yolk suspension diluted in PBST was added to appropriate wells.

Determination of activities of purified IgY: Samples of IgY extracted from egg yolks were diluted to 1,000, 3,000, 9,000, 27,000, and 81,000 times in PBST, vortexed, and centrifuged with a microcentrifuge for 2 minutes. The activity of IgY in the supernatant of each dilution was determined following the procedure described above.

Cross-reactivities of anti-P20 and anti-P100 IgY on P20 and P100 endospores: Each of the two IgY isolates (anti-P20 and anti-P100) was tested on *Pasteuria* P20 and P100 endospores. The following two ELISA procedures were used:

Procedure 1: This procedure was designed to compare P20 and P100 endospores for recognition of antibodies after adsorption of endospores onto wells. Each of the two antibodies, anti-P20 and anti-P100, were tested on endospores of *P. penetrans* P20 and P100. Following the general procedure of ELISA, endospore suspensions (50 µl) were added, starting with a concentration of 5×10^5 endospores/ml, and continuing with a series of 1:1 (v/v) dilutions. Plated antigens were exposed to antibody as a 1/10,000 dilution in PBST.

Procedure 2: This procedure was designed to compare the properties of P20 and P100 endospores to adsorb antibodies in the fluid phase. Each of the two antibody preparations, anti-P20 and anti-P100, were tested on endospores of *P. penetrans* P20 and P100. A 50-µl volume of an endospore suspension (P20 for anti-P20 or P100 for anti-P100) was added to wells of a microtiter plate. A series

of microfuge tubes was prepared containing 630 µl of 1:1 dilutions of endospore suspension in PBST, starting with 5×10^5 endospores/ml. To each, 70 µl of a 1/1,000 dilution of antibody was added. The endospore suspension was incubated with the antibodies for 1 hour (anti-P20) or 3 hours (anti-P100), and then centrifuged in a microcentrifuge for 2 minutes. Wells coated with 50 µl (1.25×10^4) endospores in PBS were then exposed to 100 µl preadsorbed or unadsorbed IgY antibody preparations.

Assay for attachment of endospores to nematodes: Second-stage juveniles (J2) of *M. arenaria* race 1 and *M. javanica* were hatched from eggs obtained from greenhouse cultures on tomato. Nematode suspensions of about 400 J2/ml were made in PBST. Endospore suspensions of P20 and P100 were made in PBST to 2×10^6 endospores/ml. Three dilutions (1/100, 1/500, 1/5,000) of IgY antibodies each of anti-P20 and anti-P100 were prepared in PBST. To each well of three columns of row A to G of a 96-well microtiter plate, 25 µl of an endospore suspension (P20 or P100) was added. To each well of a row (A to F) of the three columns, 25 µl of an antibody dilution was added. PBST without antibodies was added to row G as a control. The plate was incubated at room temperature for 2 hours before adding 50 µl of a J2 suspension to each well. The number of endospores attached per J2 was counted under an inverted microscope at ×400 magnification after incubation on a shaker for 3 hours.

SDS-PAGE analysis and immunoblot of proteins: Protein analysis was carried out using polyacrylamide gel electrophoresis (Laemmli, 1970) with a 12% separating gel. *Pasteuria penetrans* endospores (6×10^5) were placed in sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS [w/v], 10% glycerol [v/v], 0.0025% bromophenol blue [w/v], and 2% β-mercaptoethanol [v/v]) and boiled for 5 minutes at 100 °C (Davies et al., 1992). Sample lanes included prestained molecular weight markers (Bio-Rad). Following electrophoresis at constant 25 ma, gels were electro-blotted to PVDF membranes (Millipore, Bedford, MA) for protein visualization by immuno-detection with IgY antibodies directed against *P. penetrans* endospores and alkaline phosphatase-conjugated secondary anti-IgY antibodies (Sigma).

Statistical analyses: Linear regressions were performed to compare the curves of cross-activities of IgY on *P. penetrans.* Dilution numbers were transformed with $\log_{10}$ (χ + 1) before analysis. Duncan's multiple-range test was performed to compare the means of endospores attached to J2.

Results

Two hens (140-1 and 140-2) injected with P20 endospores and one hen (140-6) injected with P100 endospores yielded high levels of anti-spore activities, whereas a second hen (140-5) injected with P100 yielded a low level of activity. The activity increased rapidly after a boost injection. The highest level of anti-spore activity was observed in yolk from eggs laid at days 20 to 35 (Fig. 1).

A total of 45 ml of purified anti-P20 IgY preparation was obtained from 18 eggs laid by hen 140-1 between days 15 to 32; 25 ml of purified anti-P100 IgY was obtained from 19 eggs laid by hen 140-6 between days 22 to 34. The activities of purified IgY are illustrated in Fig. 2. Anti-P20 and anti-P100 IgY preparations exhibited similar high titers with surface antigens of *P. penetrans* P20 and P100 endospores, respectively.

Based upon ELISA, P20 and P100 endospores demonstrated comparable cross-reactivities with anti-P20 and anti-P100 IgY preparations (Fig. 3A,B), indicating predominant surface antigens are shared by both endospore types. The anti-P100 antibodies were more active in recognizing both P20 and P100 endospores than were the anti-P20 antibodies (Fig. 3A).

As shown in the immunoblot following SDS-PAGE (Fig. 4), both antibody preparations recognized a number of proteins released from each endospore type following denaturation in 2% SDS and 2% β-mercaptoethanol. Antigens common to endospores from both P20 and P100 that were recognized by the anti-P20 antibodies included those with M_r values of 23, 59, 80, and 141 KDa (Fig. 4A). Common antigens recog-

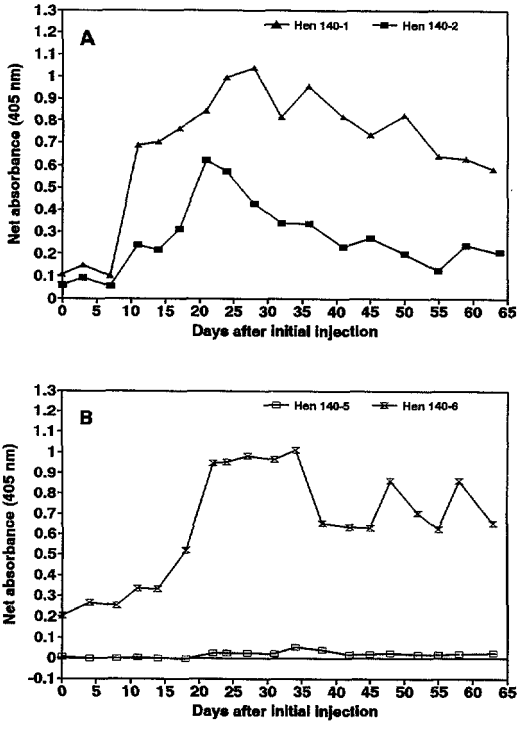

FIG. 1. Time course for the formation of IgY antispore activities. A) Two hens (hen 140-1 and hen 140-2) were injected with endospore suspension (4×10^5 endospores/ml in water) of *Pasteuria penetrans* isolate P20; 720 µl into the wing and 320 µl in the footpad. A boost injection followed 14 days after the initial injection. Net absorbance (405 nm) was recorded 30 minutes after addition of the substrate in ELISA. B) Two hens (hen 140-5 and hen 140-6) were injected with endospore suspension (4.5×10^5 endospores/ml in water) of *P. penetrans* isolate P100; 720 µl into the wing and 320 µl in the footpad. A boost injection followed 14 days after the initial injection. The absorbance was recorded at 3 hours (hen 140-5) or 30 minutes (hen 140-6) after addition of the substrate *p*-nitrophenyl phosphate di(Tris) salt solution.

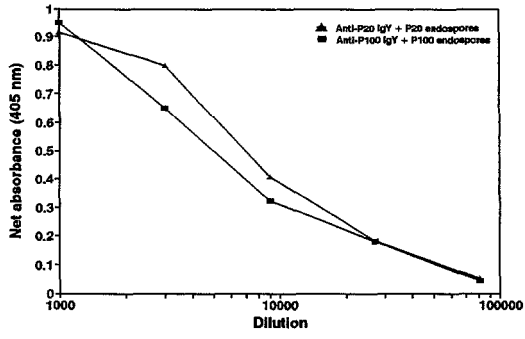

FIG. 2. Activities of purified IgY preparations. Anti-P20 IgY was extracted from yolk laid by hen 140-1 between days 15 to 32 after initial injection. Anti-P100 IgY was extracted from yolk laid by hen 140-2 between days 22 to 34. The absorbance (405 nm) was recorded at 30 minutes after addition of substrate (*p*-nitrophenyl phosphate di(Tris) salt solution).

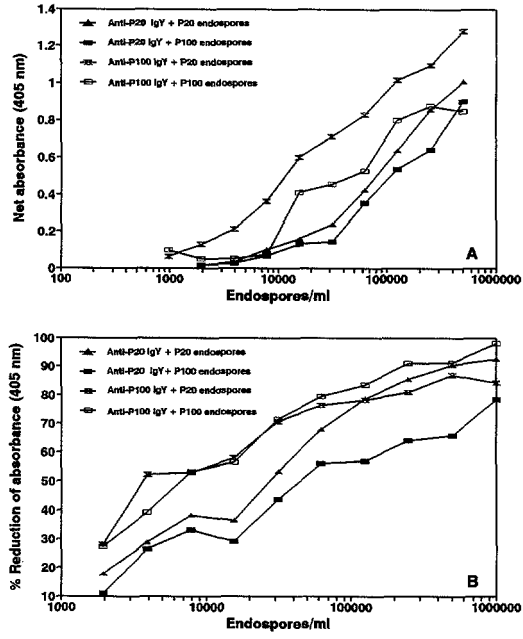

FIG. 3. Cross-reactivities of anti-P20 IgY and anti-P100 IgY on endospores of *Pasteuria penetrans* isolates P20 and P100. A) Procedure 1. A series of dilutions of *P. penetrans* endospores was added to wells of ELISA plates. Endospores that attached to the plates were incubated with a 1/10,000 dilution of purified IgY. Absorbance was recorded at 1 hour (P20 endospores) or 2 hours (P100 endospores) after addition of substrate (*p*-nitrophenyl phosphate di(Tris) salt solution). B) Procedure 2. IgY solution (1/10,000 dilution of purified IgY) was incubated with a series of dilutions of *P. penetrans* endospores for 1 hour (anti-P20 IgY) or 3 hours (anti-P100 IgY). The levels of IgY after the incubation were determined by ELISA using 50 µl of a *Pasteuria* (isolates P20 or P100) endospore suspension of 2.5×10^5 endospores/ml for each well. The percentage reduction of absorbance was based on the control.

nized by anti-P100 antibodies have M_r values of 24, 46, 57, 69, 80, 93, 113, 125, and 141 KDa (Fig. 4B). The anti-P100 with the higher avidity detected an antigen with an M_r of 62 KDa, seen only in the P100 endospores. Neither antibody preparation detected protein antigens in the 10-µg protein extracts derived from the *M. arenaria* host (Figs. 4A,B, lane 4).

After incubation of endospores with antibody, both anti-P20 and anti-P100 reduced the number of endospores attached to J2 (Table 1). The mean numbers of P20 endospores attached per J2 of *M. arenaria* race 1

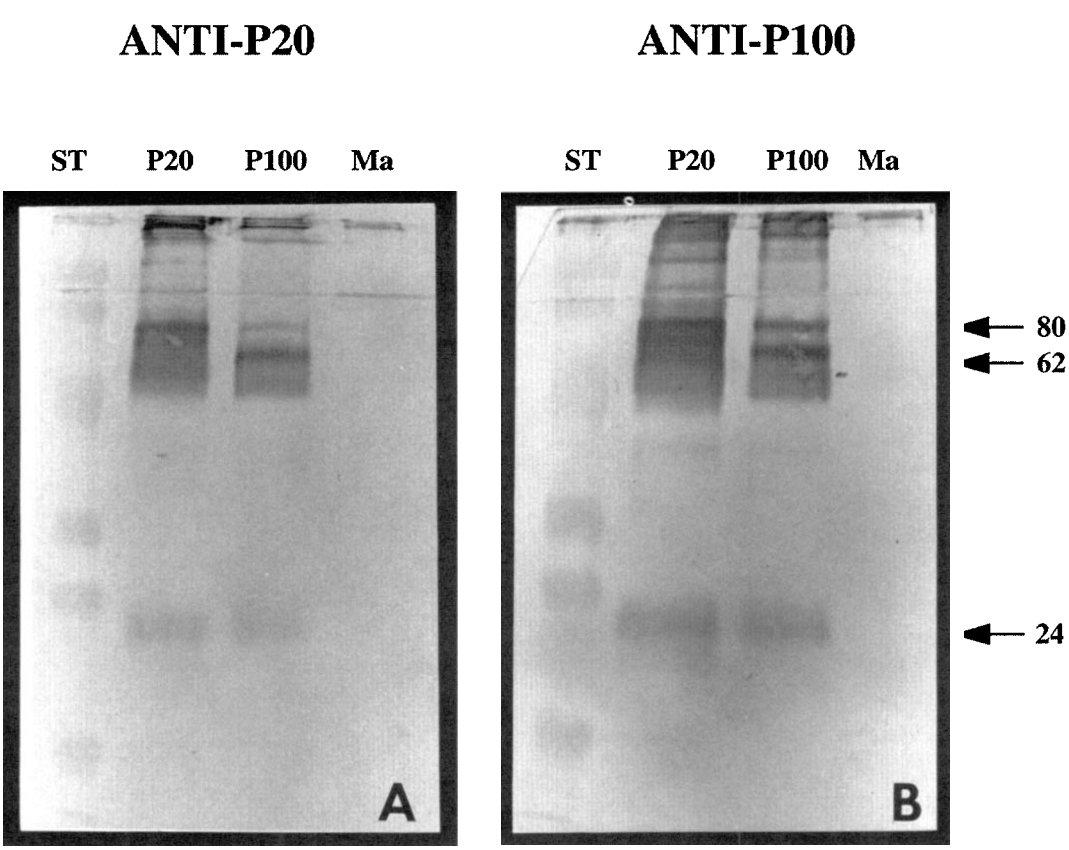

FIG. 4. SDS-PAGE and immunoblot analysis of denatured antigens extracted from endospores of *Pasteuria penetrans*. A) Detection with a 1:5,000 dilution of antibody against P20 endospores. B) Detection with a 1:5,000 dilution of antibody against P100 endospores. Lane 1: prestained markers (ST) with M_r values of 18.5, 27.5, 32.5, 49.5, 80, and 106 KDa; lane 2: 20-µl extract from 2×10^5 P20 endospores; lane 3: 20-µl extract from 2×10^5 P100 endospores; lane 4: 20-µl extract from uninfected *Meloidogyne arenaria* females containing 10 µg protein (Ma).

were 1 to 5 when the endospores were incubated with anti-P20 or anti-P100 IgY, compared to 19 endospores/J2 in the control. A consistently larger number of endospores of P100 attached to *M. javanica* J2 than occurred with P20 attaching to *M. arenaria* race 1. The mean numbers of P100 endospores attached to J2 of *M. javanica* were 20 to 30 when the endospores were incubated with anti-P20 or anti-P100 IgY, compared to 68 endospores/J2 in the control. No differences were observed between the anti-P20 and anti-P100 IgY, or among the three dilutions, on endospores from either P20 or P100.

DISCUSSION

Chickens are a useful source for antibodies against *P. penetrans* endospores. Three of the four hens injected with *P. penetrans* endospores produced a high level of anti-spore antibody. It seems that one booster injection

TABLE 1. Effect of IgY on number of endospores of *Pasteuria penetrans* (isolates P20 or P100) attached to second-stage juveniles (J2) of *Meloidogyne arenaria* race 1 (Ma1) or *M. javanica* (Mj).

Antibody	Dilution	P20 + Ma1	P100 + Mj
Anti-P20	1/200	3.5 ± 3.4 b[a]	20.6 ± 13.3 b
	1/1,000	1.3 ± 1.2 b	22.1 ± 10.4 b
	1/10,000	3.7 ± 10.1 b	21.6 ± 10.6 b
Anti-P100	1/200	5.3 ± 6.4 b	27.6 ± 17.5 b
	1/1,000	5.4 ± 8.2 b	22.9 ± 14.2 b
	1/10,000	1.5 ± 1.6 b	29.9 ± 10.2 b
Control (without antibody)		19.3 ± 12.5 a	68.3 ± 37.7 a

[a] Mean ± standard deviation, $n = 15$. The same letters in a column indicate no significant difference at $P \leq 0.01$ according to Duncan's multiple-range test.

is necessary to produce a high titer. The relative ease of using eggs versus serum as well as the low cost of animal maintenance also are important advantages.

In the cross-reactivity assays based upon ELISA, both anti-P20 and anti-P100 antibodies bound to P20 and P100 endospores. This suggests that these two antibodies shared antigens on the surface of the endospores. The denatured antigens with M_r values of 23–24, 46, and 57–59 KDa, detected in extracts from both P20 and P100 endospores with anti-P100 antibody, are similar to the sizes of those detected in other laboratories (Davies et al., 1992; Persidis et al., 1991). The antigen with M_r of 62 KDa does not correspond to any previously reported. The antigenic proteins with M_r values of 24 and 57 KDa from the P100 endospores that were reactive with both the anti-P20 and anti-P100 antibodies may account for the cross-reactivity seen by ELISA, and may be the basis for the infectivity of common hosts (Oostendorp et al., 1990). The antigen with M_r 62 KDa, which was found only in the P100 endospores, may be a component that confers a preference for a particular host, and is being carefully evaluated with respect to distribution on endospores derived from different nematode species.

Reduction of attachment of endospores to J2 indicated that at least some of the antigens were involved in recognition of endospores to the nematode. Concentrations of IgY at a dilution of 1/10,000 display a blocking activity similar to that seen at higher IgY concentrations. Complete inhibition of endospore attachment by IgY was not achieved, suggesting that chemical interactions other than antigen-antibody recognition may be involved in the attachment process as well. The incomplete inhibition seen here is in agreement with previous studies (Davies et al., 1992; Persidis et al., 1991) and may be explained by the involvement of electrostatic and hydrophobic forces documented by other studies for the attachment of endospores of *P. penetrans* to nematode hosts (Davies et al., 1996; Spiegel et al., 1996).

One or more of the antigens detected with the chicken polyclonal antibody preparation are candidates for the recognition and(or) attachment processes important for the infection of nematodes by endospores of *P. penetrans*. The large quantity and high titer of the IgY preparation make feasible the fractionation of specific antibodies through adsorption to different spore lines. This approach will be used to obtain more specific probes to identify those spore proteins most important for recognition of and attachment to the nematode host.

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