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Identification of Microsporidia infections in nature: light microscopy or PCR?

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Summary

Thelohania solenopsae is a natural pathogen of the red imported fire ant *Solenopsis invictae*, a pest widely distributed in the southern USA. Several introductions of *T. solenopsae* have succeeded in certain areas where microsporidium does not occur in assessment of *T. solenopsae* as a long-term biological control agent. A major problem in field introductions is monitoring of microsporidian infections in the release sites. The present research compared methods of detection of microsporidiosis in populations of fire ants after experimental introductions. These techniques included observation of fresh smears under phase contrast optics, Giemsa and Chromotrope-based trichrome staining, and PCR. Success/failure diagnosis by each method were compared and statistically treated by a sign test. Examination of fresh smears under phase contrast optics is not efficient and can be excluded from the diagnostic procedure. The Trichrome stain enhances diagnosis and is preferable to the Giemsa stain previously used. PCR is useful in samples that test negative by the Trichrome stain, because spores represent only a part of the complicated life cycle of *T. solenopsae,* and failure of their visualization by light microscopy can cause false negative diagnoses. A new procedure is suggested to detect microsporidiosis in field populations of insects.

Key words: Microsporidia, staining methods, molecular detection, *Thelohania*, *Solenopsis invictae*, biological control

Introduction

The red imported fire ant *Solenopsis invicta* is an introduced pest which has spread rapidly throughout the southern United States, partly due to a lack of natural enemies (Jouvenaz, 1983). A microsporidium, *Thelohania solenopsae,* was identified as a natural pathogen of *S. invicta* in South America in 1974 (Allen and Buren, 1974) and has recently been discovered in Florida (Williams et al.,1998), Texas (Cook, 2002), and Louisiana (J. F. and Y. S., unpubl.data). Ant colonies infected with *T. solenopsae* have lower fecundity and shorter life spans, suggesting that this parasite could be a promising biocontrol agent. *T. solenopsae* has been introduced successfully in certain areas where it does not occur naturally (Williams et al., 1999; J. F. and Y.S., unpubl.data) for evaluation of its potential as a biological control agent.

A major problem with field introductions is monitoring of microsporidian infection in fire ants at release sites. The current method is microscopic examination of spores either in fresh smears under phase contrast optics or after Giemsa staining of methanol-fixed smears, which results in an unacceptably high proportion of false-negative diagnoses. The recognition of microsporidiosis as one of the opportunistic infections in AIDS patients has led to development of several alternative methods to visualize microsporidia in immunosuppressed humans and in laboratory animals. Calcofluor White and Modified chromotrope-based trichrome staining have become preferred methods for this purpose (Weber et al., 1992; Van Gool et al., 1993; Didier et. al., 1995; Weber et. al.,1999; Vavra et al., 1999). The polymerase chain reaction (PCR) also is widely used for detecting microsporidia in human tissues, stool and body fluids; and this method was claimed by the authors to be 100 times more sensitive than light microscopy (Franzen and Müller,1999; Weiss and Vossbrinck,1999). Recently in laboratory experiments it was demonstrated that Trichrome and Calcoflour staining methods as well as several PCR techniques can be successfully used for revealing microsporidia in fire ants as well (Sokolova et al., 2002; Snowden et al., 2002; Valles et al., 2002; Milks et al., 2004). For *Thelohania solenopsae* PCR showed effectiveness similar to Trichrome stain and Calcofluor and much higher than Giemsa staining. The goal of this paper was to apply different detection techniques (i.e. phase contrast observation, Giemsa stain, Trichrome stain and PCR) to regular ant samples, obtained directly from the field, and to find out which one gives more positive diagnoses, and thus might be optimal in routine practice of monitoring microsporidia infections in nature.

Material and methods

Light microscopy

The ants (N=3–100) from field or laboratory colonies were homogenized in sterile distilled water in a 1.5 ml microtube with a disposable pestle (Koates Glass Co.,Vineland, NJ). Twenty µl of homogenate was spread on a glass slide over an area of 2.3 cm^2 (area of a standard cover slip) to obtain a thin transparent layer. Smears were air dried, fixed with absolute methanol for 5 min, and stained either with Giemsa[1] (Undeen, 1997) or with the modified chromotrope-based Trichrome stain (Weber et al., 1992; Didier et al., 1995). All smears were examined under a phase contrast microscope before fixation and staining.

The Giemsa stock solution was prepared as a 1.5% solution (w/v) in 100% glycerol (Undeen, 1997). The stock solution was diluted 1:40 in 0.1 M phosphate buffer, pH 7.2 immediately before use. Slides were incubated in this solution for 4-10 hours, briefly rinsed in 95% ethyl alcohol, and examined under bright-field microscope. Chromotrope 2R-based Trichrome stain was prepared according to Weber et al. (1992), with Fast Green as a counterstain.

DNA isolation and PCR

Several DNA extraction protocols and their modifications were used (Moser, 1995; Didier et al., 1996; Carville et al., 1997; Weiss and Vossbrinck, 1999), because sufficient microsporidian DNA yield has been a main concern in PCR amplifications, especially in detecting field infections at low prevalence rates. The most successful DNA isolation protocol is described: (i) the ant homogenate used in light microscopy section was diluted twice with TBS (0.1M Tris HCl, pH 7.4, 1.5M NaCl) and disrupted in a Mini beadbeater (Beospec Products, Bartleville, OK) for 30 sec at high speed with 1 mm glass beads; (ii) the contents of each tube were filtered through Kimwipes^R EX-L tissue (Kimberly-Clark, Roswell, GA) and washed with 1.5 ml of distilled water to remove potential inhibitors of DNA synthesis; (iii) the resulting suspension was centrifuged for 10 min at 8000 rpm, the supernatant was discarded, and the pellet was resuspended in distilled water (this procedure was repeated twice); (iv) the pellet was resuspended in 200 µl of TE buffer (20 mM Tris, 1mM EDTA, pH 8.0), 0.1g of 0.05 mm zirconia beads was added to each tube, and the

[1] All chemicals not designated otherwise were purchased from Sigma Chemical CO, St.Louis, MO.

suspensions were left at 95°C for 10 min and bead beaten for 1 min at low speed; (v) 300 µl of Tissue and Cell Lysis solution (Epicentre Technologies, Madison, WI), 2 µg of Lysozyme, and 3 µl of 50 µg/µl Proteinase-K (Epicentre Technologies, Madison, WI) were added, and the mixture was vortexed thoroughly and left overnight at 37°C; (vi) next day the samples were beaten again for 1 min and incubated with 1 µl of Proteinase-K and 1 µl of RNAse A (Epicentre Technologies, Madison, WI) for 1 hour at 37°C; and (vii) after cooling the samples on ice for 3-5 min, 150 µl of Protein Precipitation Reagent (Epicentre Technologies, Madison, WI) was added to each tube and vigorously vortexed. *T. solenopsae* DNA was precipitated as described in the MasterPureDNA kit protocol (Epicentre Technologies, Madison, WI) and the resulting pellet was resuspended in 25 µl of distilled water.

PCR reaction samples (25 µl) contained 10 µl of target DNA, 2.5 mM of $MgCl_2$, 0.25 mM of dNTP; 0.4 mM each of forward and reverse primers, and 1 unit of Taq DNA polymerase (Applied BioSystems, Foster City, CA). The reaction was carried out in thermocycler GeneAmp PCR System 970 (Applied BioSystems, Foster City, CA) under the following temperature profile: initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C (30 sec.), 52°C (30 sec), 72°C (1 min), and an extension step at 72°C (7 min). DNA from of *Spraguea lophii* spores was used as a positive control.

To determine the sensitivity of PCR detection dependent on the primers used, spores in ant homogenates were quantified in a hemocytometer after filtration through Kimwipes tissue. Spore concentrations were then adjusted to either 10^3 or 10^6 spores per ml. For staining tests, the approximate spore concentration was estimated directly from smears on slides.

Data were analyzed using a Sign Test (Nonparametric statistics, STATISTICA for Windows, release 5.1, StatSoft inc. 1994-1996). For statistical analysis, the data were organized in such a way that the main variables (results of Phase contrast observations, Giemsa or Trichrome staining, or PCR tests) were placed into category 1 (success, microsporidia positive) or 0 (failure, microsporidia negative). The goal of the analysis was to compare success/failure ratios of various methods, and to assess how this ratio depends on grouping variables (approximate spore concentration [ASC] and number of ants in the sample [NA]). Calculation of ASC was based on examination of Giemsa stained slides, in which we counted the average number of spores (at 1000×) in 10 microscope fields. The ASCs were initially grouped into three categories: "**1**" - no spores in ten fields (ASC $<10^4$ spores/ml); "**2**" - 1-3 spores per field (ASC = approximately 10^4 spores/ml); "**3**" - 4-10 spores in the field (ASC $\geq 10^5$ spores/ml). The NA variable also was grouped initially into three categories: "**1**" - fewer than 10 ants in the sample; "**2**" - 10-50 ants; "**3**" - more than 50 ants. Due to insufficiency of data in certain categories, the following grouping variables were used in the final analysis: ASC=1 (ASC category 1), ASC>1 (ASC categories 2 and 3 combined), NA = 1 (NA category 1), and NA>1 (NA categories 2 and 3 combined).

Results

Light microscopy

Phase contrast microscopy of fresh smears (Fig.1 A) from field and laboratory colonies revealed three types of spores: octospores, *Nosema*-like spores, and megaspores, as described previously (Sokolova and Fuxa, 2001). Occasionally, earlier stages (sporoplasms, meronts and sporonts) were identifiable as round dark bodies approximately 3-5 µm in diameter. Unfortunately, detection of the microsporidia in fresh smears failed when the concentration of spores in the sample was lower than 10^6 spores/ml. Such low resolution greatly reduces the effectiveness of this method as a diagnostic tool, because the mean concentrations of spores in the positive field samples of ants ranged from 10^3 to10^4 spores per ml.

Giemsa stain (Fig.1 B) is the primary method used to detect infections in field colonies to date. At neutral pH, microsporidia stain light blue with violet nuclei, which is similar for all microsporidia as also described elsewhere (Undeen, 1997; Weber et al. 1999). Our observations add little to the initial description of the *T. solenopsae* life cycle made by Knell et al. (1977), except for our description of megaspores (Sokolova and Fuxa, 2001).

Chromotrope 2R-based Trichrome stain, modified by Weber et al. (1992) (Fig. 1 C), was tested by us on an insect system for the first time and gave promising results (Milks et al., 2004). The walls of all spore types stained bright pinkish-red. Most of the octospores, measuring approximately 2.5 x 3.5 µm, have a distinct pinkish-stained diagonal or equatorial stripe, similar to those described for species in the genera *Enterocitozoon* and *Encephalitozoon* (Didier et al., 1995; Weber et al., 1999). Immature octospores are grouped by eight inside sporophorous vesicles, the envelopes of which stained grayish. This envelope can be disrupted easily, and usually most mature octospores are observed singly in smears. Occasionally, elongated spores, representing an adjoined pair of octospores that have failed to divide, can be seen in smears. *Nosema*-like spores, measuring approximately 2.5 x 4.5 µm, are elongated and often possess an intensively staining, pinkish-red posterior

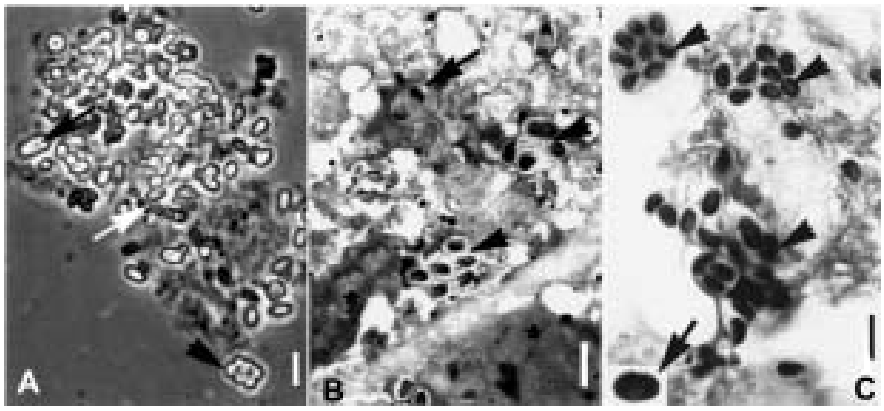

Fig. 1. Light microscopy images of tissue smears from fire ants infected with *Thelohania solenopsae*. A – fresh smear under the phase contrast optics: three types of spores are in the view; B – methanol-fixed smears stained with Giemsa stain; C – trichrome-stained smear. Arrowheads point to octospores; white arrows, to *Nosema*-like spores; and black arrows, to megaspores; asterisks indicate prespore proliferative stages of *T. solenopsae*. Scale bar:10μm.

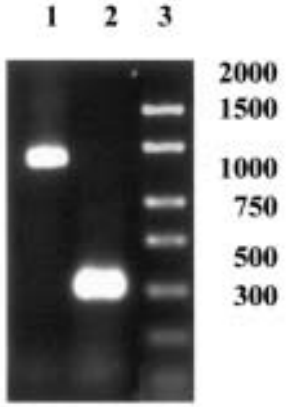

Fig. 2. PCR products of *T. solenopsae* SSU rDNA, amplified by ssV1f-1492r (line 1) and V1f-530r (line 2) primer pairs; line 3 – DNA markers (bp).

end. During diagnosis, they can be confused easily with free octospores. Megaspores, much larger (approximately 4 x 7 μm), stain more intensely pinkish-red at both posterior and anterior ends than the other two spore types, and are unlikely to be confused with the other two types of spores. Most background debris as well as bacteria and occasional mycelia of entomopathogenic fungi counterstained a faint grayish-green. Yeast cells stained dark red and did not exhibit internal structure, which allowed them to be differentiated easily from microsporidia.

Diagnosis of microsporidian infection by Polymerase Chain Reaction (PCR)

The small subunit of the ribosomal RNA gene (SSU rDNA) has been routinely selected as the target to detect microsporidia. The "universal" microsporidian primers (Weber et al., 1999; Weiss and Vossbrinck, 1999) were analyzed using the BLAST search program and alignments of published SSU rRNA gene sequences of *T. solenopsae* (Accession # AF134205, AF 031537, AF 031538; GenBank, NCBI, NIH). The best matching pairs (ssV1f-1492r and ssV1f -530r; Fig. 2) were comparatively assessed for their ability to recognize microsporidian infection. Diagnosis of microsporidiosis in fire ants with the ssV1f-ss1492r pair of primers, used previously for amplifying a ssRNA gene of *T. solenopsae* (Moser, 1995; Moser et al., 1998), was successful only if the concentration in the sample reached 10^6 spore/ml, at which point the infection could be unequivocally identified by light microscopy even without staining. On the other hand, ssV1f-ss530 were diagnostically useful and consistently successful in amplifying parasite DNA at concentrations of spores as low as 10^3 spores/ml. This pair therefore was chosen to detect microsporidia in field samples. Fig. 3 provides examples of PCR diagnosis of infection at two microsporidian release sites in Louisiana six months after introduction of *T. solenopsae* in field populations of *S. invicta*.

Comparison of effectiveness of Giemsa, Trichrome, and PCR tests for detection of microsporidian infection in field RIFA samples

One hundred fifty samples from two experimental sites were examined with phase contrast optics, by Giemsa, Trichrome, and PCR tests. *T. solenopsae* was detected by at least one of the methods in seventy-one of the samples. Sixty-one of them were evaluated by all four methods and thus were suitable for statistical analysis by the Sign Test (STATISTICA for Windows), which was applied to compare the effectiveness of the methods. Sixteen cases were identified as microsporidia-positive by phase contrast observation, 38 by Giemsa staining, 38 by PCR, and 53 by Trichrome staining (Figs 4-6). The sign test detected differences in the rate of positive diagnosis (success/failure ratio) of microsporidian infection among the four methods (Fig. 4, n=61 for all pairs). Giemsa staining was better than observation of fresh smears under phase-contrast optics (Fig. 4A, $P < 0.001$); the Trichrome stain was better than the other three methods (Figs 4B, $P < 0.001$; 4C, $P < 0.001$; 4D, $P = 0.012$); PCR was better than phase contrast observation of fresh smears (Fig. 4 E, $P = 0.001$); and the sensitivity of Giemsa staining and PCR was the same (Fig. 4 F, $P = 0.868$).

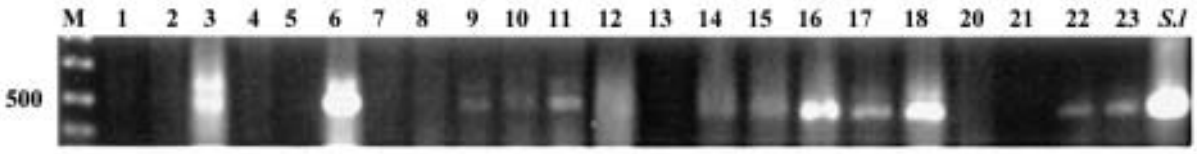

Fig. 3. PCR diagnosis of microsporidian infections in field samples with the V1f-530r primer pair. Numbers indicate colonies; M – DNA markers; *S.l* – positive control , DNA from *Spraguea lophii* spores. Samples from the experimental release site, Baton Rouge.

The numbers of ants and spores in samples affected the relative sensitivity of the four detection methods. When samples contained more than 10 ants (NA>1, Fig. 5 A, B), Trichrome staining again gave better resolution than Giemsa (n=35, P=0.004); but PCR now had the same sensitivity as Trichrome staining (n=35, P=0.343). Phase contrast observation was worse than the other three methods, and resolution by Giemsa staining remained equal to PCR (data not shown). In samples with fewer than 10 ants (NA=1), Trichrome staining was no longer more sensitive than Giemsa (Fig. 5c, n = 26, P = 0.074), and PCR was even less sensitive than Giemsa (Fig. 5 D, n=26, P=0.002). The results of comparative analyses for the other pairs remained relatively the same as in Fig. 4 (data not shown). When the concentration of spores in the sample was below the sensitivity of phase contrast observation and the Giemsa test (ASC=1, Fig. 6), the Trichrome stain detected spores successfully (Fig. 6A, n=22, P < 0.001). PCR also was successful when the number of ants in the sample was more than 10 (NA>1), although its sensitivity was not significantly greater than Trichrome staining (Fig. 6 B, n=16, P= 0.077).

Discussion

The main advantage of Giemsa staining is that it allows to detect pre-spore developmental stages. However, these stages can easily be confused with debris and cell organelles. Hence only spores possessing a characteristic staining pattern can be considered reliable for definitive diagnosis. Our data are in accord with previous observations (Weber et al., 1999) suggesting that the threshold concentration of spores which can be detected in Giemsa stained smears depends on the experience of the investigator and varies from 10^4 to 10^6 spores/ml. The main disadvantages of Giemsa staining include the intense staining background and monochrome staining of parasites in case of acid or alkaline shifts of pH of the smear, which leads to underestimation of microsporidia in positive cases.

We tested the modified chromotrope-based Trichrome stain for the first time on insect microsporidia (Sokolova et al., 2001; Milks et al., 2004). This stain is more efficient for detection of microsporidian infections in fire ant colonies than the routinely used Giemsa stain due to the bright color of spores in contrast to the weakly staining background. We tried several modifications of the Trichrome stain (reviewed in Didier et al., 1995) and found that the preparation of Weber et al. (1992) applied for 30 min at 37°C (Didier et al., 1995) leads to minimal time costs, longer preservation of the staining solution, and the greatest accuracy in spore detection. The whole staining procedure takes no longer than 1 hour; 250 ml of staining solution can be used for a minimum of 400 slides without any noticeable reduction in quality. Microsporidian infections in ant colonies were detected by Trichrome staining when the spore concentration in the suspension of homogenized ants was as low as approximately 10^3 spore/ml. Evaluation of the Trichrome stain for suspensions with known spore concentrations gave a threshold sensitivity in the range of approximately 5 x 10^2 -10^3 spores/per ml. We suggest that no fewer than 100 fields at 1000x magnification should be examined over the area of the smear (2.3 cm^2) to conclude that an infection is absent or to assess the approximate spore concentration in a sample.

Calcofluor stain showed at least the same sensitivity as Trichrome stain and its advantages and disadvantages have been described before (Milks et al, 2004). Unfortunately, because of eventual fading of fluorescence Calcofluor staining does not produce a permanently stained slides for archiving and that is why this stain was not suitable for the current project.

Purification of parasite DNA from samples is the key to utilization of PCR for diagnosis of microsporidiosis in field populations of ants, and this point was specifically addressed in our studies. Pilot experiments with estimated spore concentrations indicated that the threshold of PCR sensitivity with the "successful" pair of primers was 10^3 spores/ml, compared with 10^2 spores/ml in previous research with human microsporidia (Weiss and Vossbrink, 1999). Two major factors might cause the low rate of amplification: impurities which might contain inhibitors of DNA synthesis, or low yield of parasite DNA. Filtration of ant homogenate through Kimwipes tissue after the first bead beating followed by a water rinse definitely enhanced amplification.

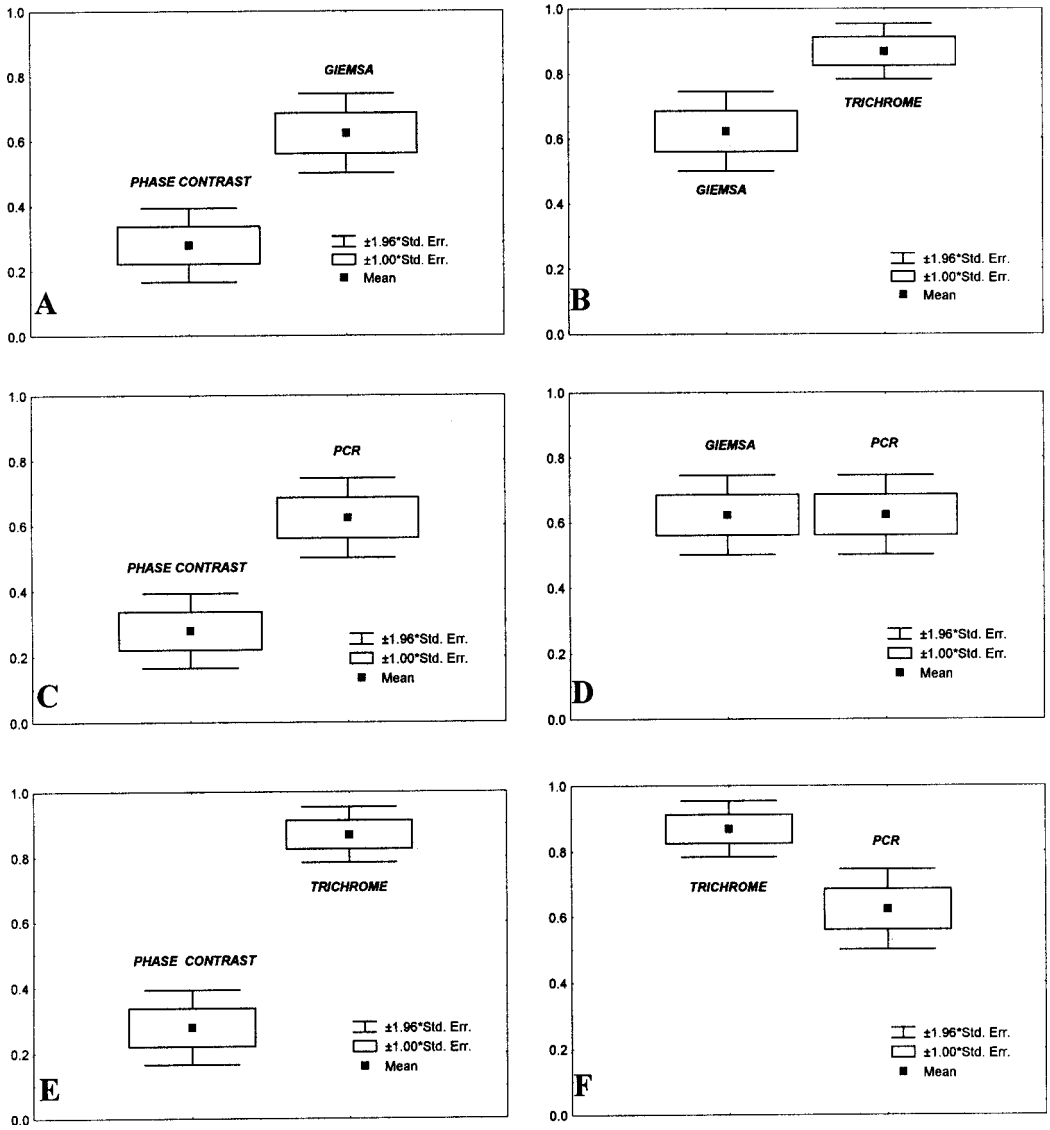

Fig. 4. Rate of positive diagnosis (Y-axis = success/failure ratio) of microsporidian infection in ant colonies by phase-contrast microscopy, Giemsa and Trichrome staining, and PCR tests among all samples (explanation in the text).

Successful PCR amplification in the absence of visible spores probably indicates that the proliferative stages, meronts and sporonts, were a major source of the DNA template. On the other hand, when spores were observed but the PCR signal was weak or absent, it is likely that DNA was not completely extracted from the spores. Thus, PCR is valuable for detecting *T. solenopsae* infection by prespore stages of the parasite, but the protocol for DNA extraction from spores must be improved.

The protocol in the current study was directed primarily at extraction of DNA from mature spores. During the preliminary spore purification from cell debris (filtration), two rounds of bead-beating with hard zirconia beads, and overnight incubation with enzymes at 37°C were evidently inadequate for purification of DNA of fragile intracellular stages closely associated with host tissues. The procedures employed here for detecting microsporidia in fire ants had been used previously (Moser, 1995; Didier et al., 1996; Carville et al., 1997; Weiss and Vossbrink, 1999) also to detect mammalian microsporidia infections in stool, urine, or body liquids, in which spores are the only or the predominant stage of the parasite life cycle. We, on the other hand, prepared samples from host tissues infected with all stages of parasite development, including fragile intracellular stages. DNA extraction from fragile

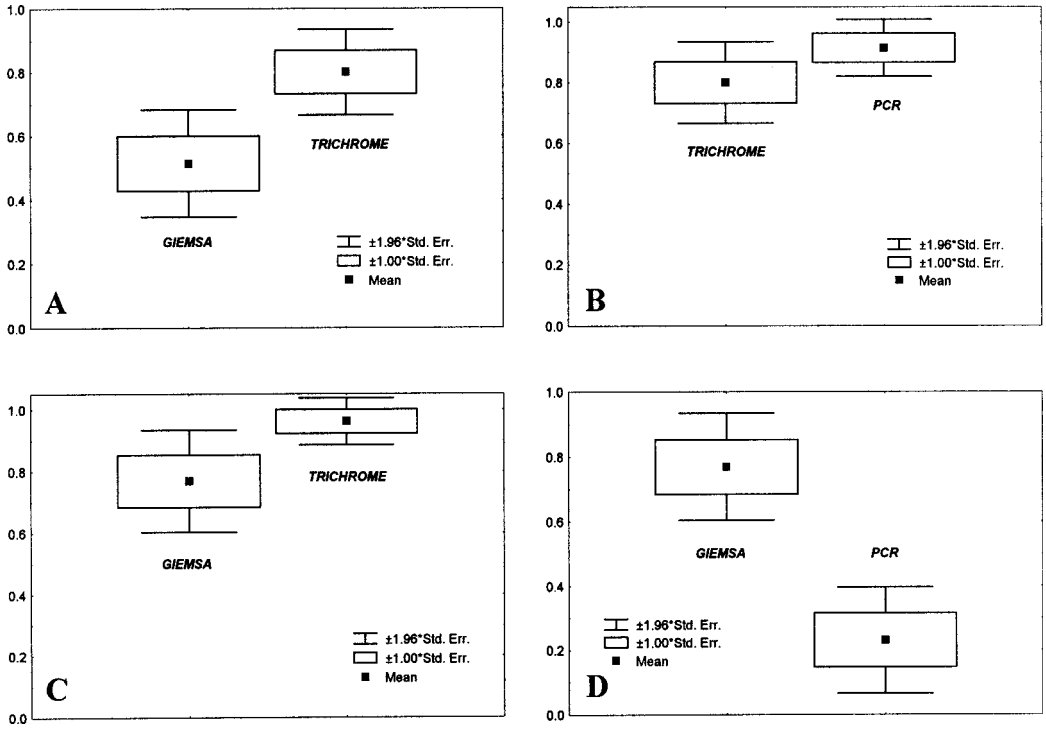

Fig. 5. Rate of positive diagnosis of microsporidian infection in ant colonies by Giemsa and Trichrome staining and by PCR in relation to the number of ants (NA) in the samples. A, B – NA>1; C, D – NA=1 (explanation in the text).

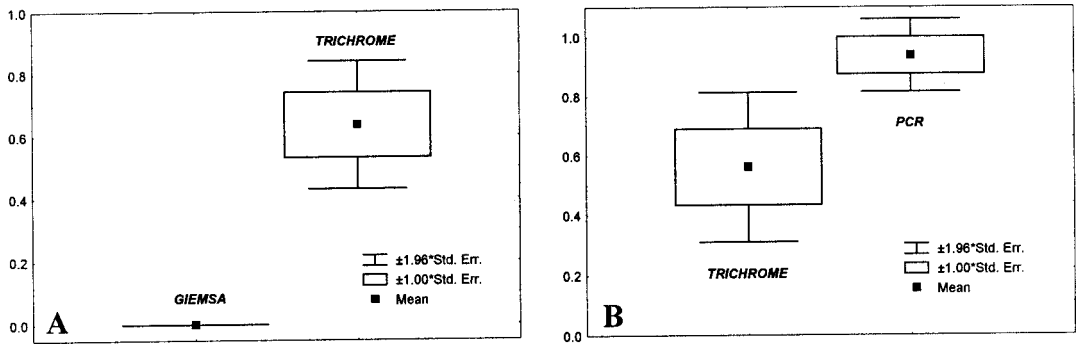

Fig. 6. Rate of positive diagnosis of microsporidian infection in ant colonies by Giemsa and Trichrome staining and by PCR in relation to the approximate concentration of spores (ACS) and the number of ants (NA) in the samples. A – ACS =1 (no spores are revealed by Giemsa staining); NA≥1; B – ACS=1; NA>1 (explanation in the text).

intracellular stages and from robust spores might demand two completely different isolation protocols alone or in combination, depending on the ratio of spores to intracellular stages in the samples.

The most stable amplification was achieved in our experiments with the ssV1-ss530 pair of primers amplifying a short product (500 bp). It is possible that damage to DNA during purification resulted in inadequate DNA template to produce the large amplicon (1300 bp), the primed by ssV1f-ss1492r oligonuclotide pair. In laboratory experiments with certain spore concentrations several alternative methods of DNA purification [FTA CARD PCR (Snowden et al., 2002); phenol-chloroform extraction (Valles et al., 2002); "crude DNA extract" (Milks et al., 2004)] also showed high sensitivity in detecting of *T. solenopsae*.

The analysis of comparative effectiveness of light microscopy and PCR techniques indicates that detection of *T. solenopasae* infection in ants may be enhanced by the use of both approaches. If spores are numerous (Figs 4, 5, 6 A), infection can be visualized through use of the Trichrome stain without PCR. On the other hand, PCR may help detect positive cases that are overlooked by light microscopy (Fig. 6 B). It is noteworthy that 13% (8/ 61) of the samples were diagnosed as positive exclusively by PCR. Optimization of DNA extraction, directed at the intracellular proliferative stages of parasite development, might overcome the limitation of the number of ants (Fig. 5 D) and increase the success rate of diagnosis.

In summary, our results suggest that staining samples with the Modified Trichrome stain (Weber et al., 1992) followed by PCR testing of Trichrome-negative samples should reduce the number of false negative cases of diagnosis of microsporidiosis in field surveys of fire ants, and probably, in other insect populations.

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