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Molecular and Biochemical Characterization of a *Coccidioides immitis*-Specific Antigen

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Results of earlier investigations have indicated that the saprobic phase of *Coccidioides immitis* produces a heat-stable, 19-kDa antigen with serine proteinase activity which has been suggested to be specific for this pathogenic fungus. In the present study we have determined the N-terminal and partial internal amino acid sequences of the purified, 19-kDa antigen, cloned the gene which encodes this polypeptide, and confirmed that the secreted proteinase is a *Coccidioides*-specific antigen (CS-Ag). Both the genomic and cDNA sequences are reported and reveal that the *csa* gene which encodes this antigen has no introns. A 543-bp open reading frame encodes a 181-amino-acid-containing protein with a predicted molecular mass of 19.8 kDa and an isoelectric point of 8.3. The *csa* gene was localized on chromosome I of three representative *C. immitis* clinical isolates on the basis of Southern hybridizations. Expression of the *csa* gene in *Escherichia coli* using the pET21a plasmid vector yielded a recombinant protein that was recognized in immunoblot assays by antibody raised to the purified 19-kDa CS-Ag. Secretion of the native antigen is suggested to occur by cleavage of a putative 23-residue signal peptide. The native CS-Ag showed a low degree of glycosylation. Analysis of the carbohydrate composition of the CS-Ag revealed xylose, mannose, galactose, and glucose. However, the purified antigen showed no affinity for concanavalin A. A PCR method with specificity and high sensitivity for detection of *C. immitis* genomic DNA, using a pair of synthetic oligonucleotide primers whose sequences were based on that of the *csa* gene, was developed. A 520-bp product was amplified only when *C. immitis* genomic DNA was used as the template. The lower limits of DNA detection using this PCR method were 1 pg of *C. immitis* genomic DNA by ethidium bromide staining and 100 fg after Southern hybridization. The *csa* gene-based PCR method for detection of *C. immitis* DNA is useful for culture identification and may have clinical applications for the diagnosis of coccidioidal infections.

Coccidioides immitis is a soilborne fungus and causative agent of a human respiratory disease known as San Joaquin Valley fever or coccidioidomycosis. The disease is endemic primarily to the desert Southwest of the United States but also occurs in parts of Mexico and Central and South America (35). Although the majority of clinical forms of coccidioidomycosis are asymptomatic or present as self-limited pneumonia, severe and progressive life-threatening coccidioidal infections which involve pulmonary and extrapulmonary tissues do occur. A recent three-year-long epidemic of valley fever in California has piqued the awareness of clinicians that *C. immitis* is a significant threat to both immunocompromised patients and healthy residents of regions of endemicity (14). Sensitive serologic tests for coccidioidomycosis are employed for separate detection of *C. immitis*-specific immunoglobulin M (IgM) tube precipitin antibody and IgG complement fixation antibody from patients with suspected or proven coccidioidal infection (36). However, alternative diagnostic procedures which do not depend on the humoral response of the patient are needed, since antibody production is sometimes delayed or attenuated (45). Conventional serologic methods are appropriate in most cases but sometimes are inadequate for diagnosis and prognosis of this fungal disease in patients with AIDS (15).

The unique parasitic cycle and distinctive morphology of *C. immitis* in vivo (9) permit diagnosis of coccidioidal infections by direct microscopic observations of draining pus isolates and occasionally by examination of sputum or bronchoalveolar lavage fluid (44). However, direct examination of clinical specimens is an insensitive test, mainly because of the small number of microbes present in most patient preparations. Clinicians usually attempt to identify *C. immitis* infections by culturing the microorganism, irrespective of the patient's immunological status. Although culturing the saprobic phase of *C. immitis* is easily accomplished, it should be performed in a biohazard containment laboratory, and confirmation of the identity of the pathogen requires in vitro conversion of the saprobic to the parasitic phase (9). Alternatively, detection of *C. immitis*-specific antigen (CS-Ag) in mycelial culture filtrates (exoantigen) of clinical isolates with commercially available antiserum typically permits culture confirmation in 2 to 3 days, using a standard immunodiffusion method (31). This exoantigen test (22) is usually conducted on 7- to 10-day-old saprobic-phase cultures and is based on reactions with positive and negative controls. The diagnostic method is highly specific if the double lines of immunoprecipitation form lines of identity with the positive control reagent (20). A CS-Ag secreted by the saprobic phase, which is at least partly responsible for the success of the *Coccidioides* exoantigen test, has been reported to be a heat-stable product (13, 23) with an estimated molecular size of 19 kDa (7, 12).

Nucleic acid probe hybridization techniques have further reduced the time necessary for fungal culture confirmation (approximately 2 h [30]) and are replacing immunodiagnostic procedures in many clinical laboratories. Commercial DNA probe assays for identification of several systemic fungal pathogens (43), including *C. immitis*, are now available (e.g., AccuProbe System; Gen-Probe Inc., San Diego, Calif.). The AccuProbe assay is based on detection of target rRNA using a

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single-stranded DNA probe conjugated with an acridinium ester moiety (21). The conjugate is hydrolyzed with hydrogen peroxide under alkaline conditions, and the chemiluminescent product is detected by A_{430}. Although the sensitivity and specificity of such DNA probes that target rRNA are high (43), some cross-hybridization which results in misidentification of fungal pathogens has been reported (31, 41). An alternative, sensitive, and rapid method for fungal identification described below makes use of the PCR technique. In this study, we characterize the 19-kDa *C. immitis* antigen, confirm that it is *Coccidioides* specific, and demonstrate that synthetic oligonucleotide primers whose sequences are based on the CS-Ag gene (*csa*) sequence can be used for detection of *C. immitis* genomic DNA by conventional PCR.

MATERIALS AND METHODS

Cultivation. The mycelial phase of *C. immitis* isolates Silveira, 634, 735, C24, UT17 to UT20, UT22, and UT24 to UT26 were grown in glucose-yeast extract liquid culture medium as previously described (33). The parasitic phase of isolate 735 was grown in a defined glucose-salts medium as reported by Kruse and Cole (25).

Isolation and purification of the CS-Ag. The 7-day mycelial culture filtrate was used as the source of the Cs-Ag. Protein components were first precipitated with cold acetone, and the pellet was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously reported (12). The 19-kDa band identified in the gel was electroeluted (12), and this fraction was used to produce a polyclonal antibody in guinea pigs as previously described (25). The antiserum was subsequently employed for immunoaffinity isolation of the CS-Ag from the same crude culture filtrate (mycelial exoantigen [ME]) by the procedure described in our earlier report (12). The bound material eluted from the immunoaffinity column was subjected to additional fractionation by high-pressure liquid chromatography (HPLC) with an ion-exchange (DEAE) column, using the method previously reported (25). The HPLC-DEAE fraction which showed maximum A_{280} was isolated, dialyzed against distilled water, and examined by SDS-PAGE for homogeneity and by immunoelectrophoresis for antibody recognition as outlined below.

IEP. Tandem two-dimensional immunoelectrophoresis (2D-IEP) (18) was used to examine the antigenic composition of the crude ME and chromatographically purified CS-Ag. Coccidioidin (CDN), which is a pooled mycelial culture filtrate plus toluene lysate of the washed hyphal fraction of several *C. immitis* strains, was used as the reference antigen. Anti-CDN (17) burro Ig was used at a 1:10 dilution of precipitated Ig reconstituted to 5 mg of protein per ml of electrophoresis buffer (barbital, pH 8.0). The reference antigen CDN (accession no. XVQ5C [8]) was added to the cathodal well at 2.5 μg/μl. Precipitin arcs were identified on the basis of the established numbering and lettering code for the CDN–anti-CDN reference system (10, 19). Rocket IEP (2) was used to examine the antigenic homogeneity of the chromatographically purified CS-Ag. The same dilution of burro antiserum as described above in of Fig. 1A was used for this latter procedure.

Amino acid sequencing. The chromatographically purified CS-Ag was subjected to SDS-PAGE (12% polyacrylamide) under reducing conditions, and the 19-kDa band was electrotransferred to an Immobilon-P membrane (Millipore Corp., Bedford, Mass.) as described by Moos et al. (29). The location of the band on the membrane was visualized by Coomassie blue staining (Bio-Rad Laboratories, Richmond, Calif.). The membrane-bound protein was excised, and its N-terminal amino acid sequence was analyzed in an Applied Biosystems model 477A gas-phase sequencer by standard procedures (16). Two different methods were used to obtain internal 19-kDa peptide sequence data. The purified CS-Ag was subjected to either cyanogen bromide digestion (CNBr; Sigma Chemical Co., St. Louis, Mo.) or enzyme digestion (endoproteinase Glu-C, endoproteinase Lys-C, or alkaline proteinase; Promega Corp., Madison, Wis.). CNBr digestion was performed according to the method of Yuen et al. (47). Enzyme digestion was conducted by using a Protein Finger-printing System kit (Promega Corp.) according to the manufacturer's protocol. The digests were subjected to SDS-PAGE (15% polyacrylamide) and electrotransferred to an Immobilon-P membrane, and the stained protein bands were excised for subsequent amino acid sequence analysis as described above.

Isolation and sequencing of the genomic clone. Degenerate PCR oligonucleotide primers were designed on the basis of selected internal amino acid sequences of the 19-kDa protein. We chose not to design a PCR primer on the basis of the N terminus of this protein because the level of redundancy was too high. The nucleotide sequences of the forward and reverse degenerate primers were as follows: forward, 5′-GAYGGNGAYAAYGGNATGAT-3′ (redundancy, 128-fold); reverse, 5′-GTCTTNGCRTTRTANGTNAC-3′ (redundancy, 256-fold). The primers were synthesized by Operon Technologies (Alameda, Calif.). The PCRs were conducted in the presence of *C. immitis* genomic DNA isolated from strain 634 or 735 as previously described (24, 33). PCR amplification was performed with a model PTC-150 Minicycler (MJ Research, Watertown, Mass.) and a Perkin-Elmer Cetus Gene Amp kit (Norwalk, Conn.). Thirty cycles were conducted for amplification of genomic DNA. Initial denaturation was performed at 94°C for 4 min. Each subsequent cycle consisted of a 94°C (1-min) melting step, a 50°C (1-min) annealing step, and a 74°C (1-min) extension. A final extension step at 74°C for 10 min was performed. After completion of the PCR cycles, 15 U of T4 DNA polymerase (Promega) was added to 100 μl of the PCR product, and the mixture was incubated at 37°C for 30 min. The PCR products were separated by agarose gel electrophoresis. A prominent DNA fragment of approximately 150 bp was isolated from the gel by using a Geneclean II kit (Bio 101 Inc., La Jolla, Calif.) and blunt-end ligated into pBluescript II KS plasmid (Stratagene, San Diego, Calif.) according to the method of Sambrook et al. (40). Sequencing of double-stranded DNA was performed by the dideoxy chain termination method (42) by using a United States Biochemical Sequenase kit as described in the manufacturer's protocol.

The PCR product was random hexamer primer labeled as previously reported (32) and was used to screen a *C. immitis* genomic library constructed in lambda Fix II (Stratagene) as described by Wyckoff et al. (46). Positive lambda clones were amplified in the *Escherichia coli* MRA strain (Stratagene), and lambda DNA was isolated from the lysate by using a lambda DNA purification kit (Qiagen, Chatsworth, Calif.). The isolated DNA was digested with *Sal*I, and the products were separated by agarose gel electrophoresis, transferred to a Zeta-Probe membrane (Bio-Rad), and hybridized with the labeled PCR product in a Southern blot as previously described (32). A 4.0-kb DNA fragment was identified and isolated, subcloned into pBluescript II KS, and subjected to bidirectional sequencing as described above. Sequence primers were synthesized on the basis of the previously determined nucleotide sequences of the 5′ and 3′ ends of the PCR product. DNA sequence analysis was performed with the DNA Strider 1.2 analytical program (28).

Isolation and sequencing of the cDNA clone. Reverse transcription-PCR (RT-PCR) was performed as described by Payson et al. (37). Total RNA was isolated from *C. immitis* according to the method of Pan et al. (33). Complementary DNAs were synthesized from 1 μg of total RNA of strain 634 or 735 with avian myeloblastosis virus reverse transcriptase (Promega) in the presence of synthesized oligonucleotide primers. The forward PCR primer was derived from the 5′ nucleotide sequence that translated an open reading frame (ORF) and showed identity to the N-terminal sequence of the 19-kDa CS-Ag (see Fig. 3). The reverse primer was an oligo$(dT)_{16}$ to which a *Bam*HI restriction site (G/GATCC) was added [i.e., 5′-AGAGAGGATCC-oligo$(dT)_{16}$-3′]. The random nucleotides added to the 5′ end of the primer increased the ease of digestion at the *Bam*HI site. Thirty cycles were conducted for amplification. Initial denaturation was performed at 94°C for 4 min. Each subsequent cycle consisted of a 94°C (1-min) melting step, a 50°C (1-min) annealing step, and a 72°C (1-min) extension. A final extension step at 72°C for 10 min was performed. The procedures of blunt-end preparation of the cDNA in the presence of T4 DNA polymerase, separation of the RT-PCR products by agarose gel electrophoresis, gel isolation of the cDNA fragment, subcloning of the cDNA into the sequencing vector, and nucleotide sequence analysis were the same as described above.

Determination of the 5′ end of the *csa* gene transcript by primer extension assay. Primer extension was conducted according to the method of Ausubel et al. (1). A synthetic 33-mer oligonucleotide primer was derived from the 5′ region of the cDNA clone (see Fig. 3). The primer sequence is as follows: 5′-CGT GCTAGCAAGTGGAGTGGCGGATGTGAAAGG-3′. The primer was labeled at the 5′ terminus with [γ-^{32}P]ATP according to the method of Sambrook et al. (40) and used to map the 5′ end of the *csa* gene transcript by primer extension. Total RNA (10 μg) was used as the template in the presence of avian myeloblastosis virus reverse transcriptase under the conditions recommended by the manufacturer (Promega). Products of this reaction were resolved by sequence gel analysis (6.0% polyacrylamide–8.0 M urea) as previously described (34).

Southern hybridization with chromosomal DNA. Chromosomal DNA of *C. immitis* was prepared and subjected to contour-clamped homogeneous electric field gel electrophoresis as previously described (32). Electrophoresis conditions were 40 V, a 75-min switch interval, and a 120-h duration at 9°C. Transfer of chromosomal DNA to a Zeta-Probe membrane (Bio-Rad) and Southern hybridization with the labeled 150-bp PCR fragment were performed as previously described (32).

Expression of the *csa* gene in *E. coli*. The *csa* cDNA (636 bp) was subcloned into the pET21a plasmid expression vector (Novagen, Madison, Wis.) which was used to transform *E. coli* BL21(DE3) by essentially the same procedure as previously reported (4). PCR amplification of the *csa* cDNA [5′ initial ATG to poly(A) tail; see Fig. 3] was conducted by using *C. immitis* total RNA with direct forward and reverse primers, each of which included *Bam*HI sites as described above. The sequences of these primers were as follows: forward, 5′-GCGGATC CATGAAGTTCTCACTCCTC-3′; reverse, 5′-AGAGAGGATCC-oligo$(dT)_{16}$-3′. The PCR conditions employed were the same as those described for the RT-PCR procedure. The PCR products were separated by agarose gel electrophoresis, and a prominent 636-bp fragment was isolated as described above. The purified PCR product was digested with *Bam*HI, reisolated and subcloned into the *Bam*HI site of the pET21a plasmid, and expressed in *E. coli* BL21 (DE3) as previously reported (4). The expression vector encoded 14 amino acids (1.4 kDa) upstream from the ATG of the *csa* gene. Expression of the recombinant protein

was induced by exposure of the transformed bacterial cells to 0.4 M isopropyl-β-D-thiogalactopyranoside (IPTG; 5-Prime 3-Prime Inc., Westchester, Penn.), using the procedure recommended by the supplier of the plasmid expression vector (Novagen). Identification of the recombinant protein was based on Western blot (immunoblot) analysis of the bacterial lysates using specific anti-CS-Ag antibody as previously described (12). The molecular size of the recombinant protein was determined by SDS-PAGE (12% polyacrylamide) with appropriate molecular weight standards.

Hydropathicity analysis of the translated *csa* gene. The plot of the hydrophobicity index of the deduced protein was generated with the DNA Strider program (28) and by the method of Kyte and Doolittle (26).

Estimation of molecular size of the deglycosylated 19-kDa CS-Ag. Because of a discrepancy between the calculated molecular size of the deduced protein and the SDS-PAGE gel estimate of the size of the native protein, we assumed that the CS-Ag was glycosylated. The monosaccharide content of the SDS-PAGE gel-electroeluted 19-kDa CS-Ag was examined by gas chromatography-mass spectroscopy (GC-MS) with authenticated, spectroscopic-grade sugar standards as previously described (6). Deglycosylation of the purified 19-kDa CS-Ag was performed by using modifications of the procedure previously reported (5). In brief, the purified and lyophilized 19-kDa CS-Ag (500 μg) was solubilized in 500 μl of 0.1 M $NaIO_4$ in 0.05 M acetate buffer (pH 4.5) and incubated at 24°C for 8 h. The reaction was arrested, and the deglycosylated product was concentrated, washed, and examined by SDS-PAGE (12% polyacrylamide) as previously described (5).

Evaluation of *C. immitis* specificity of the *csa* gene. A pair of PCR primers was designed on the basis of the *csa* cDNA sequence to evaluate whether these two oligonucleotides distinguished *C. immitis* DNA from heterologous DNA isolates. Genomic DNAs obtained from 12 different clinical isolates of *C. immitis*, 18 other pathogenic and nonpathogenic fungi, *E. coli*, and humans were tested. The list of *C. immitis* isolates and other species examined is provided in the legend to Fig. 8. The sequences of the forward and reverse PCR primers used to amplify each of the genomic DNAs were as follows: forward, 5′-AAGTTCTCACTCCTCAGCGCTATCG-3′; reverse, 5′-ACATTAAGGTTCCTCCCCTTCAACC-3′. The PCR conditions were the same as those used for synthesis of the original PCR product which was employed to screen the genomic library, except that the annealing temperature was 58°C. The predicted size of the PCR product obtained from *C. immitis* genomic DNA was 519 bp. The samples of genomic DNA were tested to determine if the primer pair amplified the same size of DNA product(s); 1 ng of template DNA was used per reaction. The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining or transferred to a Zeta-Probe membrane and hybridized with the radiolabeled 150-bp probe for Southern hybridization as described above.

The lower limit of DNA detection by PCR with the selected primer pair was tested by serial dilution of *C. immitis* (isolate 735) genomic template DNA to yield concentrations of 1 ng to 1 fg. The DNA was isolated from the mycelial phase. The limit of *C. immitis* DNA detection by PCR was also tested with template DNA extracted from viable arthroconidia (strain 735) which were added to human sputum samples obtained from a volunteer. These 'spiked' sputum samples were used in lieu of sputum from patients with coccidioidal infection. Viability of arthroconidia was determined by comparison of cell counts obtained with a hemocytometer with CFU based on glucose-yeast extract culture plate assays. The range of viable conidia added to 100-μl aliquots of sputum was 10^1 to 10^4 cells. DNA was extracted from each seeded sputum sample essentially by the procedure reported by Buchman et al. (3), except that treatment with DNase and EDTA was omitted. The *C. immitis*-containing samples were incubated with Zymolyase plus chitinase followed by proteinase K (Sigma) as previously reported (32). The isolated template DNA was amplified by PCR as described above. Agarose gel-separated PCR products were examined by ethidium bromide staining and Southern hybridization as outlined above. PCR of sputum in the presence of the oligonucleotide primers but without the addition of arthroconidia was conducted as a control.

Nucleotide sequence accession number. The GenBank accession number for the 19-kDa CS-Ag gene (*csa*) is L36551.

RESULTS

Isolation of the CS-Ag from the ME. The pattern of precipitin peaks shown in the 2D-IEP gel in Fig. 1A is typical of the electrophoretic separation and immunoprecipitation of CDN components under the conditions described. Four of the precipitin peaks are labeled according to the established reference system (10, 19). Both antigen 2 and *C. immitis*-specific antigen precipitins are clearly deflected when the reference antigen is subjected to 2D-IEP in tandem with the 7-day ME (ME 7; Fig. 1B). These two antigens are prominent in the mycelial culture filtrate of *C. immitis*. A major polypeptide component of the ME was previously revealed by SDS-PAGE to have a molecular size of 19 kDa and identified by tandem 2D-IEP as the CS-Ag (12). The 19-kDa protein was purified from the ME 7 by a series of separation steps which included acetone precipitation, electroelution, immunoaffinity, and HPLC with an ion-exchange DEAE column. The chromatographically purified product was added to the tandem, cathodal well of the 2D-IEP plate (Fig. 1C). Only the *C. immitis*-specific antigen precipitin component of the reference antigen showed a tandem shoulder. The results of rocket IEP with this same chromatographic fraction added to the cathodal wells at increasing concentrations are shown in Fig. 1D. Single precipitin peaks, which are indicative of antigenic homogeneity, were revealed above each well.

Protein sequencing and PCR. The purified 19-kDa CS-Ag (Fig. 2A) was isolated and subjected to N-terminal sequence analysis. The 33-residue sequence of the N terminus is shown in Fig. 2B. Additional internal amino acid sequence data were obtained from peptides produced by CNBr digestion and proteolysis of the 19-kDa protein (Fig. 2A). The N-terminal sequence of the major CNBr-derived peptide (approx. 6.5 kDa) is presented in Fig. 2B. Lys-C and Glu-C endoproteinase digestion yielded prominent 4.5- and 8.0-kDa peptides, respectively, whose N-terminal amino acid sequences showed overlap with that of the CNBr fragment (Fig. 2B). The 4.0-kDa product of alkaline proteinase digestion showed a distinct amino acid sequence.

The internal protein sequence data were used to design a pair of degenerate PCR primers. A 146-bp PCR product was obtained with this primer pair and *C. immitis* genomic DNA as the template. The deduced amino acid sequence of the oligonucleotide product (underlined in Fig. 3) was identical to the protein sequence reported in Fig. 2B. The labeled PCR product was used as a probe to isolate the *csa* gene from the *C. immitis* genomic library.

Nucleotide and deduced amino acid sequence analysis of the *csa* gene. The DNA sequence shown in Fig. 3 includes a 543-bp ORF, which encodes a 181-amino-acid polypeptide. The calculated molecular size of the translated protein is 19.8 kDa, and its isoelectric point is 8.3. The translated sequence of the ORF includes the previously described N terminus of the native protein and four peptides obtained by CNBr and proteolytic digestion of the 19-kDa protein (Fig. 2B). A cDNA clone corresponding to the entire predicted coding region of the gene was obtained by RT-PCR as described above. The cDNA was entirely sequenced, and it proved to be identical to the genomic DNA sequence. No introns were present in this *C. immitis* gene. The primer extension assay resolved the position of the terminal Met and the putative transcription start point. The latter corresponds to nucleotide (nt) −125 from the AUG start codon (Fig. 3). The nt −125 is located 28 nt downstream from a putative transcriptional promoter sequence, TATATA, in the 5′ untranslated region. The cDNA sequence identified the location of the stop codon, the putative poly(A) site (TATAA), and the initiation site for the poly(A) tail (nt 620) in the 3′ untranslated region of the *csa* gene.

Southern blot analysis of *C. immitis* chromosomal DNA. Southern blot analysis of chromosomal DNA components, performed with the labeled 146-bp PCR product, showed hybridization to chromosome I in the three clinical isolates of *C. immitis* examined (Fig. 4).

Expression and recognition of the recombinant CS-Ag. SDS-PAGE separations of the lysates of *E. coli* BL21(DE3) are shown in Fig. 5. The lysate derived from bacteria which were transformed with the pET21a plasmid plus the 636-bp cDNA insert (pET21aCS) and induced with IPTG revealed a distinct Coomassie blue-stained band of approximately 21 kDa. Western blot analysis of this same lysate using the CS-Ag-specific

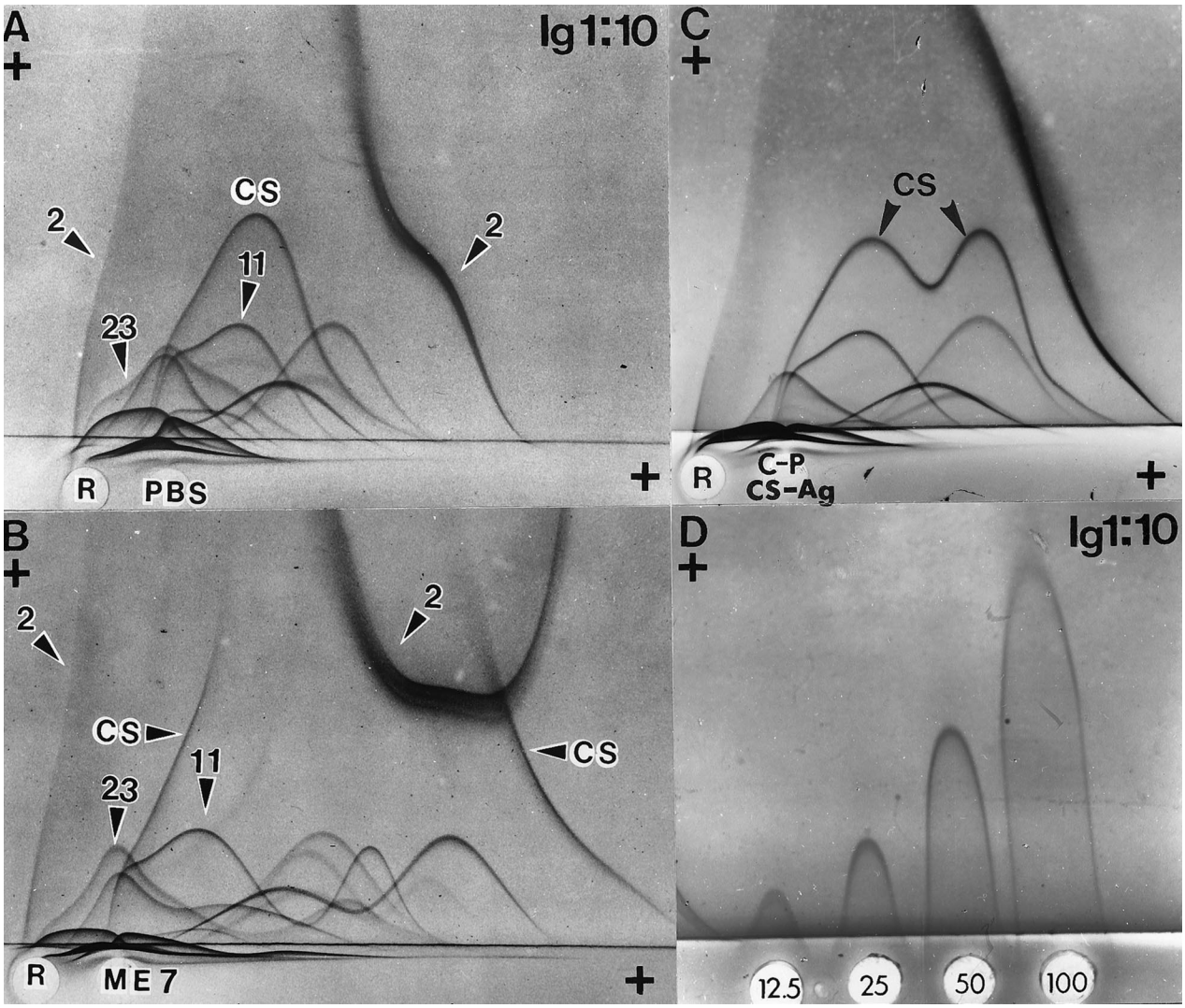


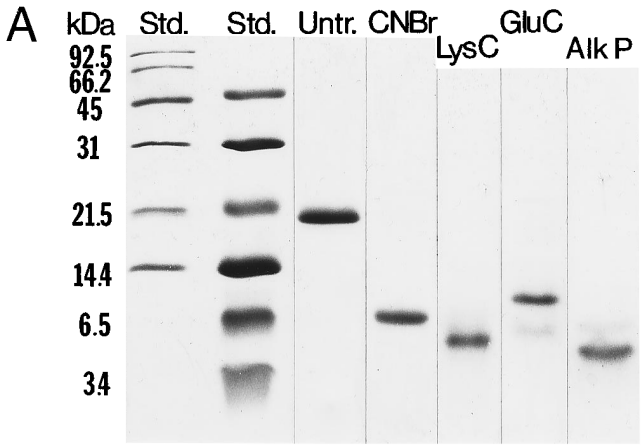
FIG. 1. Tandem 2D-IEP (A to C) and rocket IEP (D) of the reference antigen (CDN [R]) and *C. immitis* antigens against burro Ig. The dilution of Ig contained in the upper gel (above horizontal line in figures) was 1:10 in electrophoresis buffer. (A) The tandem wells contain CDN (R) and phosphate-buffered saline (PBS). (B and C) The cathodal wells contain the 7-day ME (ME 7 at 100 μg/ml of PBS) and the chromatographically purified CS-Ag (C-P CS-Ag at 20 μg/ml of PBS), respectively. (D) Concentrations of the purified CS-Ag in the cathodal wells are indicated (in micrograms per milliliter of PBS). The plus sign indicates anodes and the direction of migration in each dimension. Numbers in panel A represent antigenic components of CDN which are also present in ME 7 (B).

antiserum recognized a single 21-kDa band (Fig. 5). As described above, the recombinant protein includes a 1.4-kDa fusion peptide encoded by the vector. The estimated molecular size of the recombinant *C. immitis* protein, therefore, is 19.6 kDa, which approximates the size of the deduced protein encoded by the *csa* gene.

Hydropathicity profile. The plot of the hydrophobicity index of the deduced protein (Fig. 6) suggests that the junction of residues 23 and 24 (Fig. 3) is a putative cleavage site between the signal peptide and the mature, exported protein. This junction corresponds to the N-terminal amino acid of the mature protein as determined by sequence analysis (Fig. 2B). On this basis, the calculated molecular size of the signal peptide is 2.4 kDa, while that of the mature protein is 17.5 kDa.

Deglycosylation of the CS-Ag. Because of the difference between the calculated molecular size of the deduced, mature protein and the molecular size of the native protein based on SDS-PAGE examination, we assumed that the 19-kDa CS-Ag was glycosylated. However, no potential sites of N-glycosyl linkages were evident from the deduced protein sequence (Fig. 3). The monosaccharide composition of the methanolyzed and derivatized 19-kDa CS-Ag was determined by GC-MS (Fig. 7). Mannose and glucose were the major components, with trace amounts of xylose and galactose. The ratio of monosaccharides, based on integration of methylglycoside peaks of the GC, is 2.8:44.9:3.2:49.2 for xylose-mannose-galactose-glucose. Complete deglycosylation of the 19-kDa CS-Ag with 0.1 M $NaIO_4$ was confirmed by GC-MS analysis of the treated sample. SDS-PAGE separation of the desalted and concentrated deglycosylation product revealed a single, silver-stained band with a molecular size of approximately 17.5 kDa (inset; Fig. 7). The size of the deglycosylated CS-Ag is the same as the predicted molecular size of the mature protein.

Detection of *C. immitis* genomic DNA by PCR with *csa* primers. A product of approximately 520 bp was amplified by PCR with the synthesized oligonucleotide primer pair of the *csa*



B

Amino acid sequence analysis

N-terminus	ASTADLSYDTHYDDPSLPLSGVTCSDGDNGMITKGYN
CNBr digestion	ITKGYNTAGEIPNYPHVG
Lys-C endoproteinase digestion	GYNTAGEIPNYPHVGGAFTVET
Glu-C endoproteinase digestion	IPNYPHVGGAFT
Alkaline proteinase digestion	NSPNCGKCYKVTYNAKTIFL

FIG. 2. (A) SDS-PAGE (15% polyacrylamide) separation of purified CS-Ag (Untr.) and major peptide components of CS-Ag after digestion with CNBr, endoproteinase Lys-C, endoproteinase Glu-C, and alkaline proteinase (Alk P). The bands were visualized by Coomassie staining. Std., standards. (B) Amino acid sequences determined for the N terminus of untreated CS-Ag and the peptide components of the digests cited above.

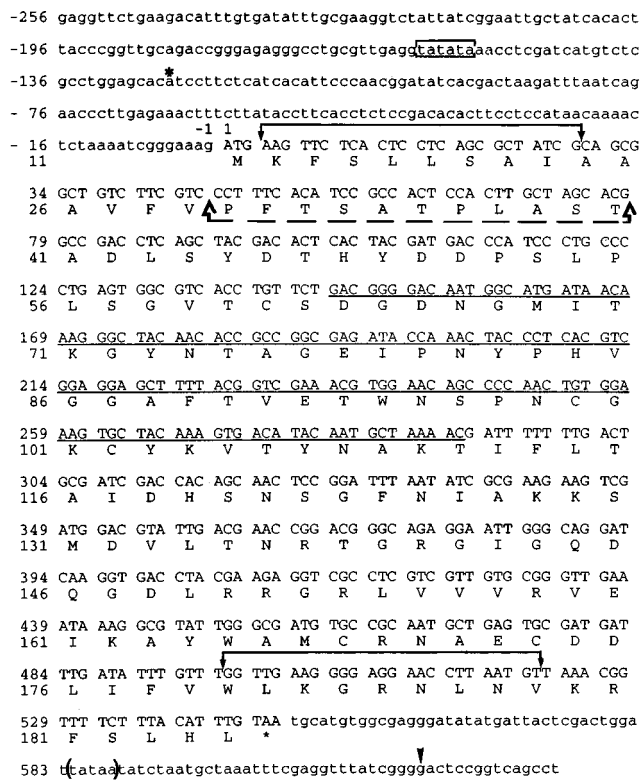


FIG. 3. Nucleotide sequence of *C. immitis csa* gene and deduced amino acid sequence of CS-Ag. The solid underlined nucleotide sequence represents the 146-bp PCR product. The dashed underline with arrows indicates the primer used for primer extension. The two solid lines with arrows above the nucleotide sequence bracket the 5′ and 3′ primers used in PCR amplification of the 519-bp *C. immitis*-specific *csa* gene fragment in the presence of genomic DNA of the pathogen. The asterisk in the 5′ region indicates the putative transcription start point (bp −125). The putative transcriptional promoter consensus sequence is boxed. The asterisk in the 3′ region is the stop codon, and the parentheses mark the putative poly(A) site. The arrowhead (bp 620) indicates the putative initiation site for the poly(A) tail.

gene (Fig. 3) and template genomic DNA of all 12 clinical isolates of *C. immitis* tested (Fig. 8A). However, none of the other template genomic DNA preparations from pathogenic or nonpathogenic fungi, *Pneumocystis carinii*, *E. coli*, or *Homo sapiens* yielded a 520-bp PCR product. The list of heterologous fungi tested included members of the *Onygenaceae* (*Ascomycetes*), which have been shown to be phylogenetically related to *C. immitis* (33). No amplification was observed without *C. immitis* template DNA. No difference in length of the PCR product was observed between the 12 clinical isolates of *C. immitis*. The specificity of the PCR amplification using the *csa* primer pair was confirmed by Southern analysis (Fig. 8B).

Results of evaluation of *C. immitis* genomic DNA detection and sensitivity are shown in Fig. 8C and D. PCR with the selected primer pair of the *csa* gene was able to detect 1 pg of *C. immitis* genomic DNA by ethidium bromide staining (Fig. 8C) and 100 fg by radioisotope labeling (Fig. 8D). PCR conducted with the DNA extracted from sputum samples seeded with *C. immitis* arthroconidia detected 10^2 cells by ethidium bromide staining and 10^1 cells by Southern hybridization (data not shown). No amplified product was obtained from sputum samples lacking *C. immitis* arthroconidia.

DISCUSSION

The CS-Ag was originally identified by tandem 2D-IEP (8) as a component of CDN, a crude skin test reagent used to evaluate delayed-type hypersensitivity response to *C. immitis* exposure in humans (17). CS-Ag was not one of the 26 antigens reported by Huppert and coworkers (18, 19) in their compositional analysis of CDN based on 2D-IEP separations. Advancing-line IEP (8) was used to test for the presence of the CS-Ag in culture filtrates, cell wall, and cytosol fractions of different *C. immitis* cell types, as well as in similar preparations from other pathogenic and nonpathogenic fungi. The results suggested that the CS-Ag is a secretory product of both the saprobic and parasitic phases of *C. immitis* and is apparently unique to this respiratory pathogen (9, 10). We previously demonstrated (12) that the CS-Ag is a component of the heat-stable antigen used by Kaufman and coworkers (23) for immu-

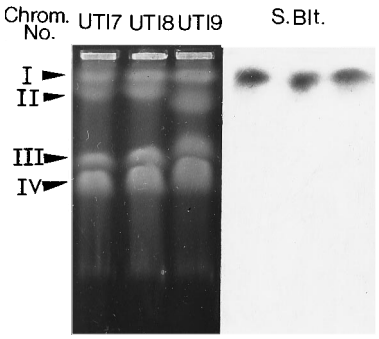


FIG. 4. Ethidium bromide-stained contour-clamped homogeneous electric field electrophoresis gel (40 V, 75-min switch interval, and 120-h duration at 9°C) of three designated isolates of *C. immitis* and autoradiograph of the membrane-transferred chromosomal DNA hybridized with the labeled 146-bp *csa* gene probe. Chromosome I is labeled for each isolate. S. blt., Southern blot.

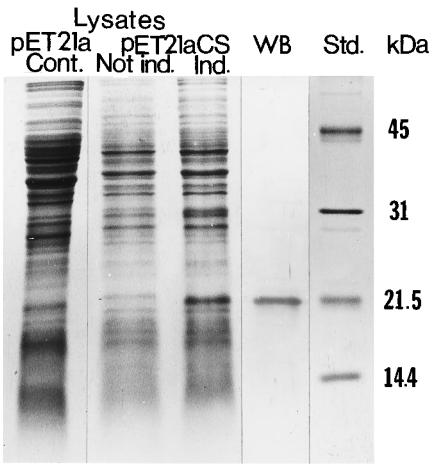


FIG. 5. SDS-PAGE (12% polyacrylamide) separations of lysates of transformed *E. coli* BL21(DE3) and Western blotting (WB) with antibody raised against the native CS-Ag. The control lysate (Cont.) was derived from transformants with the plasmid (pET21a) alone. Lysates of transformants with the recombinant plasmid (pET21aCS) were obtained from *E. coli* before and after IPTG induction (Not ind. and Ind., respectively). The antibody recognized an approximately 21-kDa component only in the lysate derived from the pET21aCS transformant after IPTG induction. The bands were visualized by Coomassie staining. Std., standards.

noidentification of *C. immitis* cultures. The purified CS-Ag was recognized by the antiserum employed in this exoantigen immunodiffusion assay (7). Because of the apparent unique and immunogenic properties of the CS-Ag, it was anticipated that detection of anti-CS-Ag antibody in patient sera could be used for diagnosis of coccidioidal infection. Serum samples from 21 coccidioidomycosis patients and 21 control donors from hospital admissions with no reported systemic or pulmonary mycoses were tested for IgG antibody reactivity with the purified 19-kDa CS-Ag by enzyme-linked immunosorbent assays (ELISAs). A broad range of antibody reactivity was observed, and several serum samples from patients with confirmed *C. immitis* infection were negative by ELISA. This was not surprising, since Kaufman et al. (23) had previously reported that sera from patients with coccidioidal infections were infrequently reactive with the reagent used in their exoantigen test. Unlike the tube precipitin and complement fixation antigens of *C. immitis* (17), therefore, the pathogen-specific exoantigen is of little serodiagnostic value. On the other hand, immunoelectron-microscopic studies using antibody raised to the purified antigen have revealed that the CS-Ag is secreted in vivo from parasitic cells. Small amounts of CS-Ag-specific label were detected, however, suggesting that in vivo and in vitro expression of the antigen are not comparable. Evidence that the 19-kDa CS-Ag is a proteinase, with optimal activity at pH 6.0 and 37 to 40°C, has been presented (7, 12). The 19-kDa proteinase is apparently tolerant of a broad range of conditions (pH 4.0 to 8.0 and 24 to 60°C). Results of enzyme inhibitor studies suggested that the CS-Ag is a member of the class of trypsin-like serine proteinases (7). Determination of substrate preference revealed that the purified CS-Ag is capable of efficient in vitro digestion of IgG and secretory IgA at pH 7.2 (7). It is possible that low levels of secretion of the CS-Ag from parasitic cells at sites of infection, combined with proteolysis of host Igs in the presence of the fungal proteinase, may contribute to the variation in antibody titers to this antigen observed in ELISAs of patient serum samples.

The objective of this study was to confirm that the 19-kDa polypeptide detected in the culture filtrate of the saprobic phase is indeed a CS-Ag. Our strategy was to isolate the *csa* gene which encodes the CS-Ag and then use PCR methods to evaluate the specificity of this gene as a probe for detection of *C. immitis* DNA. The antigen was isolated from saprobic-phase cultures, and homogeneity of the chromatographically purified secretory product was confirmed by both SDS-PAGE and rocket IEP. A 37-residue, N-terminal sequence of the antigen was obtained, which further attests to the purity of the isolated protein. A homologous N-terminal sequence was reported by Resnick and coworkers (39) for an 18- to 21-kDa serine pro-

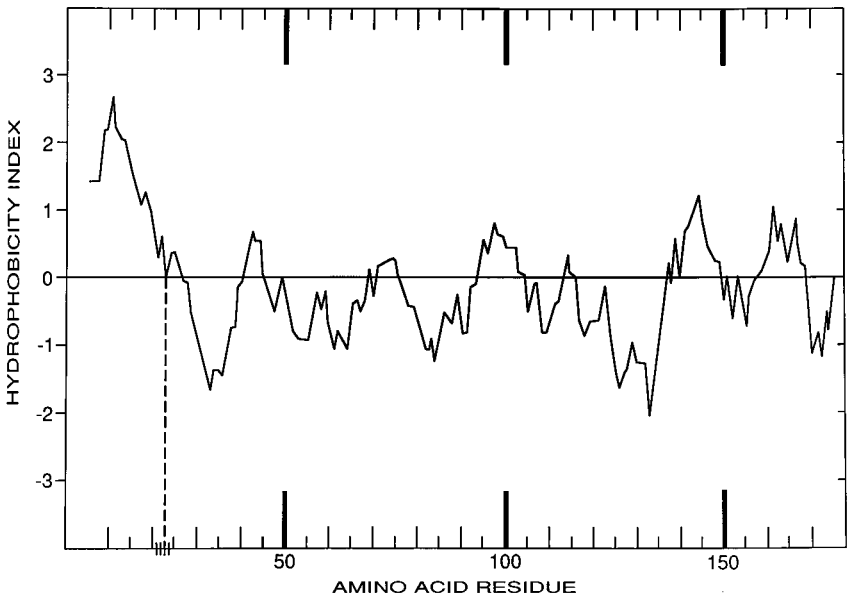


FIG. 6. Hydropathic profile of the deduced CS-Ag protein. The vertical dashed line indicates residue 23 (leucine). It is suggested, on the basis of the N-terminal sequence data (Fig. 2B), that the putative signal sequence cleavage site is at the carboxyl side of residue 23. Refer to Fig. 3.

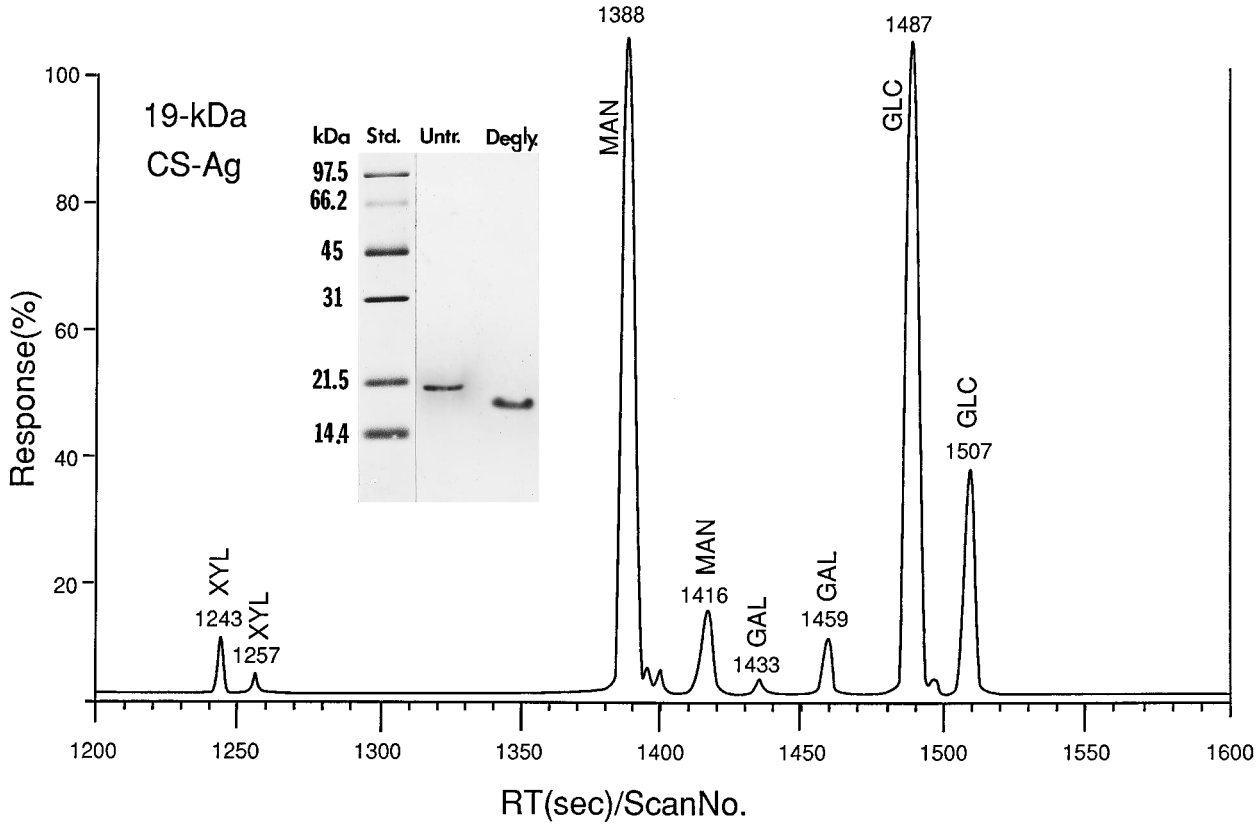


FIG. 7. Gas-liquid chromatograph of trimethylsylated methylglycosides of monosaccharides present in the purified 19-kDa CS-Ag. Peaks are labeled as follows: XYL, xylose; MAN, mannose; GAL, galactose; GLC, glucose. Inset shows SDS-PAGE (12% polyacrylamide) separation of untreated, purified CS-Ag (Untr.) and $NaIO_4$-treated, deglycosylated CS-Ag (Degly.). Std., standards.

teinase of *C. immitis* with possible elastinolytic activity (38). The enzyme was isolated from the spherule culture filtrate of the Silveira strain of *C. immitis*. The 25-residue sequence (T-P-L-S-T-A-D-L-S-Y-D-T-H-Y-D-D-P-S-L-P-L-S-G-V-T) reveals near identity (underlined) to our N-terminal sequence of the 19-kDa CS-Ag shown in Fig. 2B. The only sequence difference is the addition of three residues at the N terminus which may reflect variation between the two strains examined. Digestion of the purified 19-kDa CS-Ag by incubation with CNBr or selected proteinases yielded peptides which were then isolated and subjected to amino acid sequence analysis. These data permitted us to design a pair of degenerate oligonucleotide primers that were used in PCR with *C. immitis* genomic DNA for isolation of a product which could be used to screen the genomic library.

Both the genomic and cDNA sequences of the *csa* gene were determined. The locations of the terminal Met, putative transcription start point, and initiation site for the poly(A) tail were resolved and supported our conclusion that the *csa* gene lacks introns. This is in contrast to other reported genes of *C. immitis* which contain introns of approximately 50 to 90 bp (34, 36). The *csa* gene was located on chromosome I in the three clinical isolates examined and appears to be a single-copy gene (data not shown). The cDNA contained a single ORF which includes 543 bp that encode a predicted protein of 181 amino acids with a molecular mass of 19.8 kDa. The 636-bp cDNA which was subcloned into the pET21a plasmid expressed a recombinant bacterial protein of approximately 21 kDa. Taking into account a 1.4-kDa fusion peptide encoded by the expression vector, the estimated molecular size of the recombinant protein (19.6 kDa) is comparable to the predicted molecular size of the protein encoded by the entire ORF. However, the CS-Ag is a secreted protein with a predicted signal peptide that includes 23 amino acids (2.4 kDa). Evidence for the signal peptide is based on both the N-terminal sequence data and the hydropathicity profile. The predicted molecular size of the mature protein after subtraction of the signal peptide is 17.5 kDa. The difference between the predicted and SDS-PAGE estimates of the molecular size of the secreted CS-Ag (approx. 1.5 kDa) is suggested to be due to glycosylation of the protein. In the absence of conserved amino acid sequences which would indicate N-glycosyl linkages (11), it is probable that the sugars are attached to the protein core by O-glycosyl bonds. In spite of the presence of mannose and glucose residues, as indicated by GC-MS analysis of the purified 19-kDa CS-Ag, the glycoprotein failed to bind to concanavalin A (7). Cox and Britt (13) described a heat-stable, species-specific exoantigen of *C. immitis* which was isolated from the unbound effluent fraction of a concanavalin A affinity column separation of the mycelial culture filtrate. Although the molecular size of the exoantigen was not reported, we suggest that this heat-stable antigen is equivalent to the CS-Ag. Possible explanations for the apparent lack of affinity between the 19-kDa CS-Ag and concanavalin A may be the low carbohydrate content of the glycoprotein and/or the nature of presentation of the sugar residues.

Results of the PCR method for detection of *C. immitis* DNA using an oligonucleotide primer pair derived from the *csa* gene sequence showed amplification of an identical 520-bp product in all 12 selected clinical isolates of the pathogen. The level of sensitivity of this DNA detection method is comparable to that

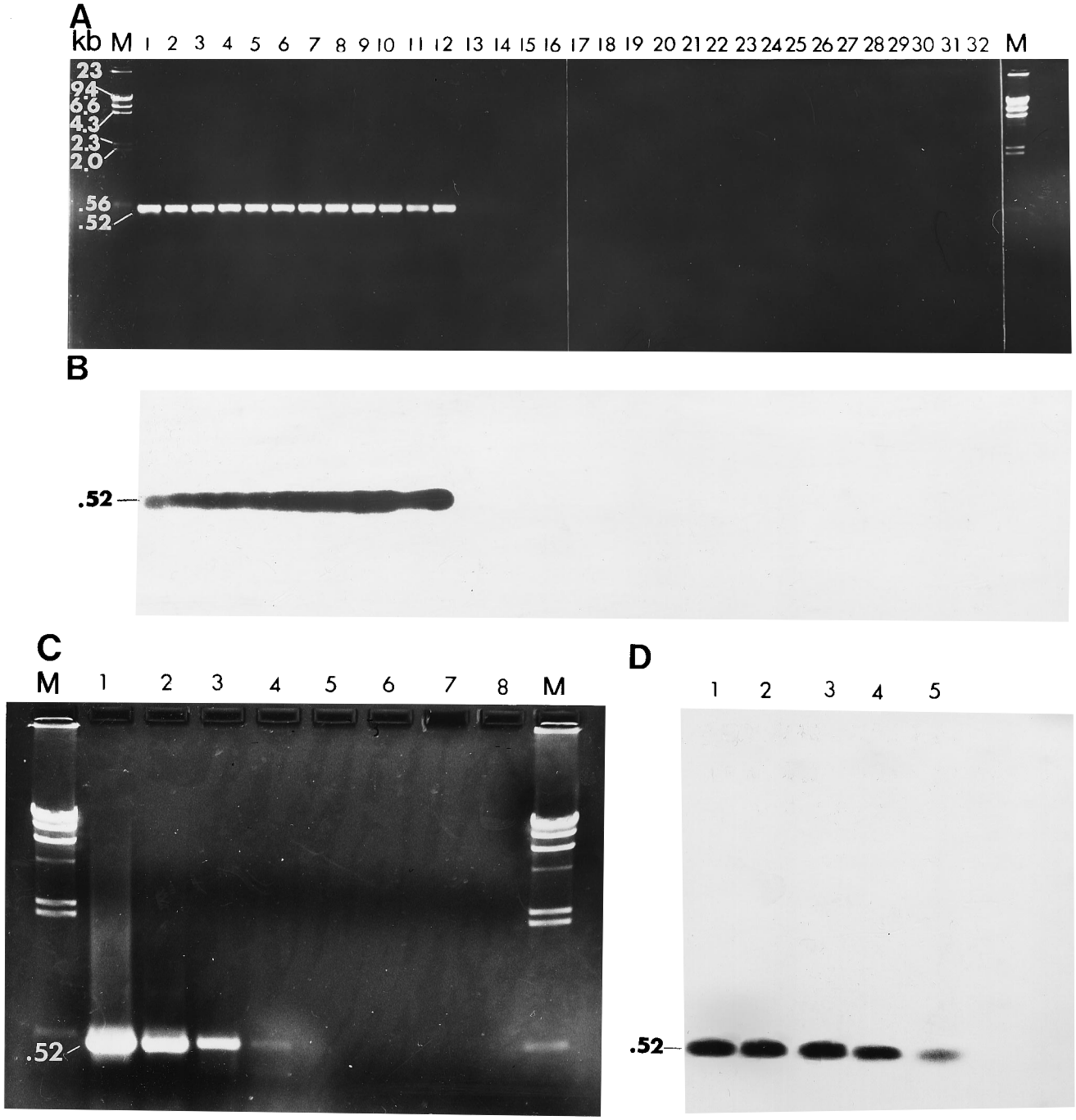


FIG. 8. Specificity (A and B) and sensitivity (C and D) of the PCR with the selected *csa* gene-derived oligonucleotide primer pair (Fig. 3). (A and B) Agarose (1.5%) gel electrophoresis of PCR products amplified from 1.0 ng of genomic DNA templates from various organisms. (A) Gel stained by ethidium bromide which shows 519-bp PCR product of near equal intensity in each *C. immitis* isolate; (B) Southern hybridization of membrane-transferred DNA products shown in panel A with labeled 146-bp *csa* gene probe. Lanes 1 to 12, clinical isolates of *C. immitis* (735, 634, Silveira, UT26, UT25, UT24, UT22, UT19, UT18, UT17, UT20, and C24, respectively); lane 14, *Uncinocarpus reesii*; lane 15, *Malbranchea dendritica*; lane 16, *Malbranchea filamentosa*; lane 17, *Candida albicans* CA30; lane 18, *C. albicans* CA87; lane 19, *Pneumocystis carinii*; lane 20, *Wangiella dermatitidis*; lane 21, *Aspergillus flavus*; lane 22, *Aspergillus parasiticus*; lane 23, *Cryptococcus neoformans*; lane 24, *Histoplasma capsulatum* G217B; lane 25, *H. capsulatum* UT; lane 26, *Blastomyces dermatitidis* UT; lane 27, *Aspergillus fumigatus*; lane 28, *Paracoccidioides brasiliensis*; lane 29, *B. dermatitidis* 26197; lane 30, *E. coli*; lane 31, *Saccharomyces cerevisiae*; lane 32, *Homo sapiens*. (C and D) Agarose (1.5%) gel electrophoresis of PCR products amplified from different amounts of *C. immitis* isolate 735 genomic DNA template. (C) Gel stained by ethidium bromide; (D) Southern hybridization of membrane-transferred DNA products shown in panel C with labeled 146-bp *csa* gene probe. (C) Lanes 1 to 8 show results of amplification of genomic template DNA as follows: 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and none, respectively. M, *Hin*dIII-digested lambda DNA. The molecular size range (M in panel C) is the same as for panel A.

of reported methods in which the primer pair was derived from nucleotide sequences of fungal 18S rDNA genes (27). In preliminary studies we have recently shown that the *csa* primer-based PCR method can achieve these same levels of specificity and sensitivity of *C. immitis* DNA detection with crude DNA extracts of infected lung tissue of BALB/c mice. Clinical evaluations are necessary to further test the efficacy of this *C. immitis* DNA detection method with specimens of patient blood, cerebrospinal fluid, and sputum.

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