Counterimmunoelectrophoresis in the Detection of Antibodies Against *Coccidioides immitis*

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In recent years counterimmunoelectrophoresis has been increasingly used in the diagnosis of microbial diseases, and our study was undertaken to evaluate its possible use in the rapid detection of antibodies against *Coccidioides immitis*. One hundred twelve specimens from 50 patients were studied. The study population comprised the following: Group I, 34 patients with an active coccidioidomycosis infection; Group II, five patients with a positive coccidioidin skin test; Group III, five patients with a negative coccidioidin skin test; and Group IV, six patients with other fungal infections. Counterimmunoelectrophoresis was positive in 93 of 96 specimens in Group I and negative in the other groups. The results obtained by counterimmunoelectrophoresis correlated well with complement fixation and double immunodiffusion and indicate that it is an excellent tool for the diagnosis of patients with coccidioidomycosis.

IN RECENT YEARS counterimmunoelectrophoresis has emerged as one of the most promising diagnostic tools in infectious diseases, its simplicity making its application feasible even in countries with underdeveloped laboratory technology (1). Since the description of its use in the detection of hepatitis B surface antigen (HBsAg) (2), successful applications of counterimmunoelectrophoresis have been reported in the detection of antigens or antibodies in viral (3, 4), bacterial (5-9), and parasitic (10-13) diseases. In the diagnosis of mycotic infections, it has been shown to be useful in the detection of antibodies in cases of histoplasmosis (14), candidiasis (15), aspergillosis (16), coccidioidomycosis (17), and paracoccidioidomycosis (18).

The diagnosis of coccidioidomycosis in endemic areas is based on its clinical manifestations, recent conversion of a known negative skin test to positive, isolation of the organism from secretions or surgical specimens, and specific serologic tests. The consideration of this infection as a diagnostic possibility in nonendemic areas is essential in the early recognition of the disease.

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Complement fixation, tube precipitin, latex particle agglutination, and double immunodiffusion are the serologic methods that have been used in the diagnosis, follow-up, and prognosis of acute, chronic, and disseminated forms of infection with *Coccidioides immitis* (19-21). There is a 100% correlation of combined complement fixation and double immunodiffusion results with an active or recent infection in patients with a confirmed infection and normal humoral immunity (21). However, the cost, time consumption, and technical difficulties of the complement fixation method are factors against its systematic application in routine laboratories, which necessitates the referral of clinical specimens to large specialized laboratories. Although the double immunodiffusion method is simple to perform and is sensitive and specific, it may take 24 to 72 h for the complete development of the reaction. In addition, it has not been adopted as a quantitative test for coccidioidal antibodies.

In this study we evaluated the use of counterimmunoelectrophoresis in the detection of antibodies against *C. immitis* and found that the results correlated well with complement fixation and double immunodiffusion as a tool in the diagnosis, follow-up, and prognosis of patients with this infection.

Materials and Methods

CLINICAL SPECIMENS

One hundred twelve specimens (67 sera, 27 spinal fluids, and 18 ventricular fluids) from 50 patients were evaluated. Group I from the study population included 34 patients with active coccidioidomycosis infections (96 specimens). Three of these patients were seen at Milwaukee County General Hospital: one had an acute self-limiting disease (seven sera); one a chronic pulmonary form (four sera); and one the disseminated form of the disease with meningeal involvement requiring the intrathecal and intraventricular administration of amphotericin B (19 sera, 18 spinal fluids, and 17 ventricular fluids). One patient with histiocytic lymphoma seen at the Veterans Administration Hospital (Wood, Wisconsin) developed a chronic pulmonary coccidioidal infection (one serum). Another 30 specimens (20 sera, nine spinal fluids, and one ventricular fluid) from other patients with active infection (nine with acute infections, three with chronic pulmonary infections, and 18 with disseminated disease, in 10 of whom the dissemination was to the meninges), had been received by one of us (D.P. at the University of California; Davis, California). The remainder of the study population comprised the following: Group II, sera of five patients with a positive (1:100) coccidioidin skin test

(Cutter, Berkeley, California) without signs of a systemic infection; Group III, sera of five patients with a negative (1:100) coccidioidin skin test without signs of a systemic infection; and Group IV, sera of six patients with fungal infections other than *C. immitis*. This group included one case each of pulmonary blastomycosis, mucor endocarditis, rhodotorula fungemia, *Torulopsis glabrata* fungemia, cryptococcal meningitis, and systemic candidiasis. Diagnosis in each of these cases was made by clinical manifestations, isolation of the organism, and appropriate serologic tests.

ANTIGEN

Spherulin (Berkeley Biologicals, Berkeley, California), a commercial antigen prepared from the spherules of *C. immitis* and purchased as a 1:8 dilution (for use in the complement fixation test), was used in the detection of antibody by counterimmunoelectrophoresis and double immunodiffusion. The optimal dilution of the antigen was defined as the dilution that gave the highest antibody titers with a specific standard serum; this was found to be 1:8 (or undiluted) for both tests. Spherulin and mycelial antigen (culture filtrate plus autolysate [22]) were used as antigens in the complement fixation test.

COUNTERIMMUNOELECTROPHORESIS

Counterimmunoelectrophoresis was done according to the method described previously (5) using a continuous buffer system. A plastic mold was placed on an 82 mm × 102 mm projector slide cover glass plate (Eastman Kodak Co., Rochester, New York) and flooded with 14 ml of a 1% agarose (Marine Colloids, Inc., Rockland, Maine) solution prepared in 0.075 M barbitone buffer, pH 8.6. Parallel rows of wells, 3 mm in diameter, were cut 3 mm apart. Patients' sera and cerebrospinal fluid (CSF) were placed in the wells nearest to the anode and the antigen preparation in those nearest to the cathode. Each clinical specimen was tested from an undiluted sample through 1:64 against the antigen dilution of 1:8. Electrophoresis was carried out for 90 min with the powerpak set at 475 V, which resulted in a current of 25 mA across the plate. The plates were immediately read, then placed in a 0.9% sodium chloride solution overnight and observed again the following morning. There was no difference in the sensitivity of the method between the two readings, although increased delineation of the reaction was observed 12 h after the initial reading. The last dilution of serum at which a precipitin reaction could be detected was defined as the precipitin titer. An example of a positive serum by counterimmunoelectrophoresis is shown in Figure 1.

DOUBLE IMMUNODIFFUSION

Double immunodiffusion was carried out using 82 mm × 102 mm projector slide glass plates. A plastic mold was placed on the glass and flooded with 14 ml of a 1% agarose solution in 0.075 M barbitone buffer, pH 8.6. Each cover glass contained 16 five-well patterns, each well being 3 mm in diameter and 3 mm apart. The pheripheral wells were filled with the patient's serum or CSF from an undiluted sample through a 1:16 dilution. The center well was filled with a 1:8 dilution of the spherulin. The plates were incubated in a moist chamber at room temperature and examined for the presence of precipitin lines at 24, 48, 72, and 96 h. The test was interpreted as positive if a precipitin reaction was present at any one dilution. An example of a positive serum by double immunodiffusion is shown in Figure 2.

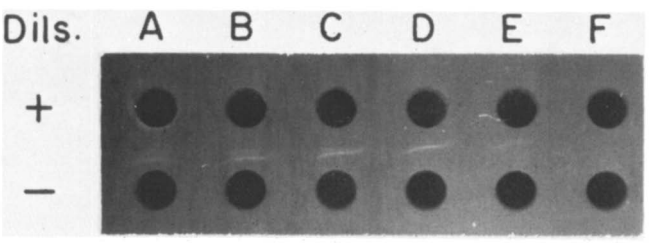

Figure 1. Demonstration of serum coccidioidal precipitins by counterimmunoelectrophoresis. Sera dilutions were placed in wells on the anodal (+) side and undiluted spherulin on the cathodal (—) side. The following serum dilutions (*dils*) were used: A, undiluted; B, 1:2; C, 1:4; D, 1:8; E, 1:16; and F, saline.

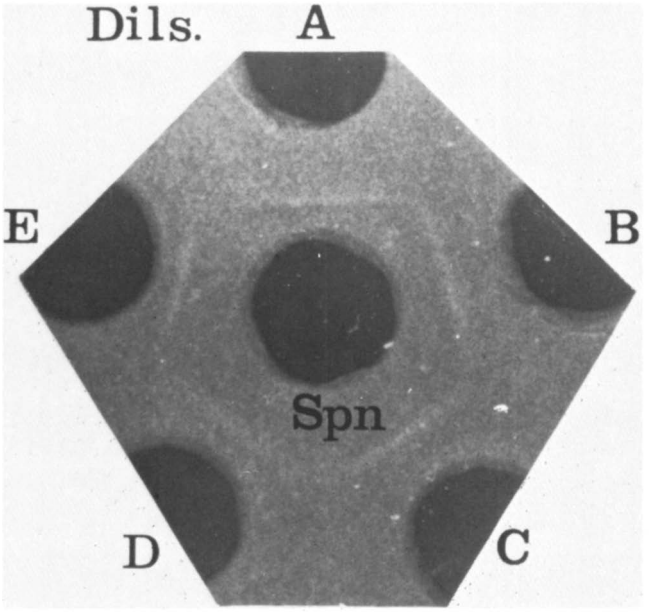

Figure 2. Demonstration of coccidioidal precipitins by double immunodiffusion, formed by serum dilutions (*dils*) of a patient infected with *Coccidioides immitis*, run against spherulin (*spn*), dilution 1:8. The following serum dilutions were used: A, undiluted; B, 1:2, C, 1:4, D, 1:8; and E, 1:16.

COMPLEMENT FIXATION

Complement fixation was done at the University of California at Davis using mycelial coccidioidin and spherulin. Each specimen was tested at different dilutions starting with a 1:2 dilution. Occasionally because of small amounts of sample, it was necessary to start with a 1:4 dilution. Binding of complement for a 2-h period at 37 °C was used for sera and overnight (18 h) at 4 °C for the CSF specimens. Results obtained with coccidioidin were used in the comparison with results obtained by counterimmunoelectrophoresis and double immunodiffusion.

Results

The results of antibody detection by all three methods for the four groups are summarized in Tables 1 and 2. In Group I, a patient with histiocytic lymphoma and hypogammaglobulinemia was negative by the three methods. He had a chronic pulmonary form of coccidioidomycosis. Two other patients with meningitis with complement fixation titers in the CSF of 1:2 and 1:4, respectively, were negative by counterimmunoelectrophoresis and double immunodiffusion.

False-positive reactions were not observed in any of the groups with the counterimmunoelectrophoresis and double immunodiffusion methods (Table 1). Lines of identity were observed between clinical specimens (serum and spinal fluid) from patients with an active *C. immitis* infection and the reference-positive serum using spherulin as antigen. No precipitin reactions were observed in any of the six sera of patients with other mycoses (Group IV), nor in normal human serum.

Table 1. Detection of *Coccidioides immitis* Antibodies by Complement Fixation, Counterimmunoelectrophoresis, and Double Immunodiffusion

Group	Clinical State	Complement Fixation		Counter-immunoelectrophoresis		Double Immunodiffusion	
		Number Tested	Number Positive	Number Tested	Number Positive	Number Tested	Number Positive
I	*C. immitis* infection	96	95	96	93	96	93
II	Coccidioidin skin test (+) without sign of infection	5	0*	5	0	5	0
III	Coccidioidin skin test (−) without sign of infection	5	0*	5	0	5	0
IV	Other mycotic infections	6	1*	6	0	6	0

* Anticomplementary activity: Group II, three sera; Group III, four sera; and Group IV, three sera.

Anticomplementary activity was shown in three of five specimens of Group II, in four of five specimens of Group III, and in three of six sera of Group IV. An additional serum from this group showed a positive complement fixation titer of 1:8 (patient with mucor endocarditis).

Figure 3 shows good correlation between the results of antibody titration obtained by complement fixation and counterimmunoelectrophoresis on 96 specimens in Group I. The results of complement fixation and counterimmunoelectrophoresis antibody titers of 34 patients in the same group, according to the clinical form of infection, are summarized in Figure 4. Complement fixation titers in the primary form of the disease were in the range of 1:4 to 1:32, whereas titers of 1:2 to 1:16 were generally obtained by counterimmunoelectrophoresis. In the chronic pulmonary form the complement fixation titers were generally in the range of 1:8 to 1:32 and by counterimmunoelectrophoresis, 1:4 to 1:16. In the disseminated form without meningitis, serum complement fixation titers were in the range of 1:16 to 1:128 and by counterimmunoelectrophoresis, 1:2 to 1:64. In the group with meningeal dissemination, complement fixation titers in the CSF were in the range of 1:2 to 1:16 and by counterimmunoelectrophoresis, undiluted to 1:16.

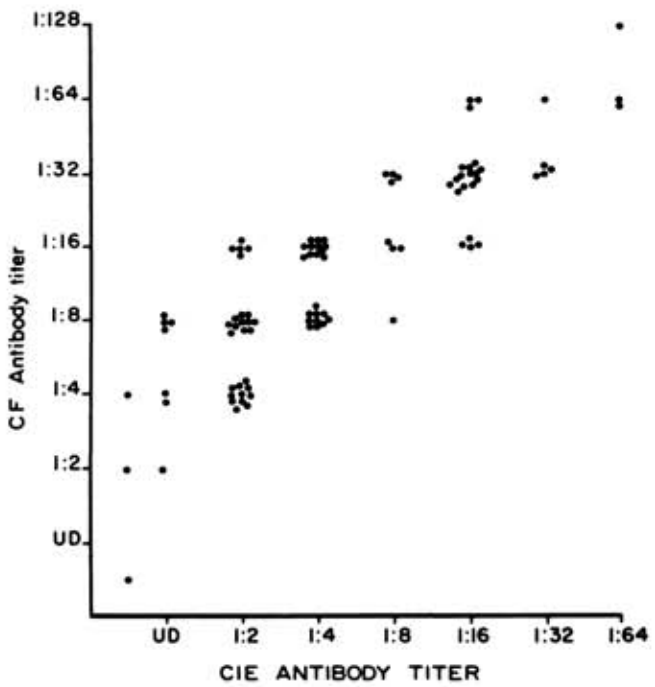

Figure 3. Complement fixation (*CF*) antibody titers of all 96 clinical specimens (sera and cerebrospinal fluid) in Group I plotted against counterimmunoelectrophoresis (*CIE*) antibody titers; UD = undiluted.

In three patients—one with an acute self-limiting disease, one with a chronic pulmonary form, and one with meningitis—a serologic follow-up was possible for periods of 11, 7, and 12 months, respectively. Good correlation between both tests was found during the period of time in which the follow-up was carried out.

Complement fixing activity was evaluated with the use of mycelial-coccidioidin and with spherulin antigens. In general, the titers obtained with spherulin were comparable or one dilution higher than the ones obtained with coccidioidin. Anticomplementary activity was more frequently observed when spherulin was used.

Discussion

Coccidioidomycosis is an endemic infection of the New World. It occurs in the Northern Hemisphere in areas that correspond to the ecologic concept of the lower Sonoran Life Zone (that is, semiarid climate, alkaline soil, hot and dry summers followed by a short period of rain [23, 24]). Active forms of the disease can be acute (San Joaquin fever, Valley fever), chronic, or disseminated infections. Complement fixation and double immunodiffusion detect antibodies against *C. immitis* in the three forms of the disease (19-21). However, in patients with impaired humoral immunity a lack of normal production of antibodies in response to infectious agents will be present. The diagnosis in these patients can be obtained by the isolation of the fungus in secretions or surgical specimens. In our study the only patient with active disease who showed negative serology by the three methods had a histiocytic lymphoma with undetectable levels of immunoglobulins.

Counterimmunoelectrophoresis is a method that is simple to do and does not require specialized personnel or equipment. The time required for a complete reaction is 30 to 90 min. Because of these factors, this method could be used in small hospitals in countries lacking sophisticated laboratory facilities (1). Counterimmunoelectrophoresis has been successfully used in the detection of antigens or antibodies in viral (hepatitis-associated antigen [2] and influenza A [3, 4]); bacterial (*Streptococcus pneumoniae* [5, 6], *Neisseria meningitidis* [7], *Hemophilus influenzae*

Table 2. Detection of *Coccidioides immitis* Antibodies by Complement Fixation, Counterimmunoelectrophoresis, and Double Immunodiffusion in Group I Patients

Form of the Disease	Number of Patients	Complement Fixation (+)	Counterimmunoelectrophoresis (+)	Double Immunodiffusion (+)
Primary	10	10	10	10
Chronic pulmonary	5	4	4	4
Disseminated	8	8	8	8
Meningitis (CSF)*	11	11	9	9
Total	34	33	31	31

* CSF = Cerebrospinal fluid.

type B [7], *Pseudomonas aeruginosa* [8], and *Staphylococcus aureus* [9]); parasitic (*Entamoeba histolytica* [10, 11], *Trichinella spiralis* [12], and *Trypanosoma cruzi* [13]); and fungal infections (*Histoplasma capsulatum* [14], *Candida albicans* [15], *Aspergillus fumigatus* [16], *Coccidioides immitis* [17], and *Paracoccidioides brasiliensis* [18]). Correlation of this method with complement fixation and double immunodiffusion in the detection of antibodies against *C. immitis* was found to be 98% (93 of 95) and 100% (93 of 93), respectively, in clinical specimens of patients with the active form of the disease and with normal immunologic response. Only two patients with meningitis and CSF titers of 1:2 and 1:4 by complement fixation were negative by counterimmunoelectrophoresis and double immunodiffusion. Positive reactions were not found by counterimmunoelectrophoresis or double immunodiffusion in patients with positive coccidioidin skin tests without signs of systemic infection (Group II) or in patients with negative coccidioidin skin tests without signs of systemic infection (Group III). In the same groups, complement fixation showed anticomplementary activity in three of five patients (Group II) and in four of five patients (Group III). These results indicate that counterimmunoelectrophoresis in addition to complement fixation and double immunodiffusion is highly sensitive and specific in detecting active forms of the infection (19, 20).

Cross-reactivity between *C. immitis, Blastomyces dermatitides,* and *H. capsulatum* antigen in the complement fixation test is well known (21). Clinical specimens with low or high antibody titers against *C. immitis* may cross-react with the heterologous antigens; conversely, sera from other systemic fungal infections may cross-react with coccidioidin antigen. In the group with other fungal infections (Group IV), one patient with mucor endocarditis had a positive complement fixation test, with a titer of 1:8, and three more showed anticomplementary activity. The possibility of cross-reactions between some bacterial antigens and carcinoantigens with *C. immitis* antigen has been suggested (21), but on the basis of much other experience this is questionable (19).

On the other hand, double immunodiffusion, a qualitative test, has been shown to be as sensitive and more specific than complement fixation; it correlates better with true coccidioidomycosis infections when complement fixation titers are in the range of 1:2 to 1:8 (21). In this series, 98% of the clinical specimens from serologically proved cases (complement fixation) of coccidioidomycosis infections (Group I) were positive by double immunodiffusion, and no cross-reactions were observed with clinical specimens from patients with fungal infections other than coccidioidomycosis (Group IV). Because of the combination of high sensitivity and lower specificity of complement fixation in clinical samples with low titers, and the high specificity of double immunodiffusion in the same group, it has been recommended that both these tests be carried out simultaneously on patients with suspected coccidioidomycosis infections to avoid any possible misinterpretation of results and delay in the correct diagnosis of an active *C. immitis* infection.

Our results indicate that counterimmunoelectrophoresis is an excellent method for the detection of antibodies in *C. immitis* infections. It was shown that counterimmunoelectrophoresis is as sensitive as complement fixation and double immunodiffusion (98 and 100% correlation, respectively) and as specific as double immunodiffusion. The use of the new antigen spherulin, a soluble extract from autolyzed spherules of *C. immitis,* may have played a role in the improvement of the sensitivity of counterimmuno-

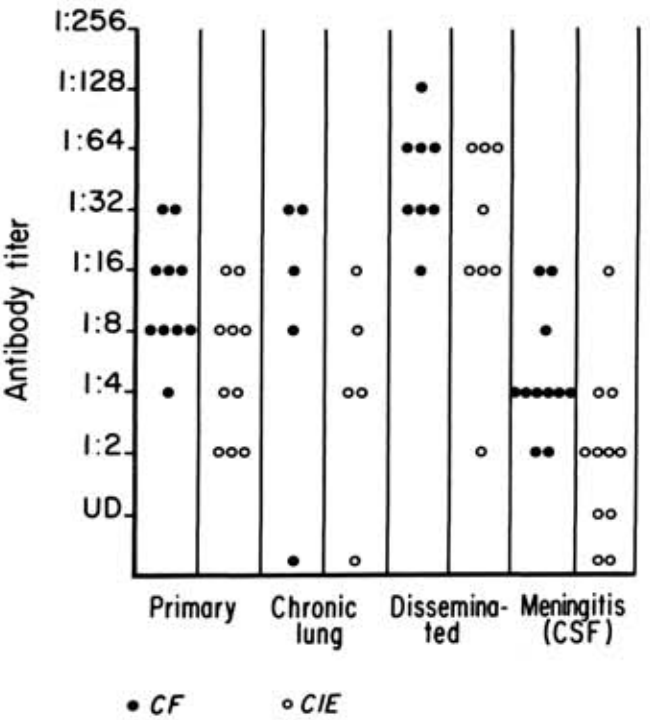

Figure 4. Correlation of antibody titers by complement fixation (*CF*) and counterimmunoelectrophoresis (*CIE*) in 34 patients with different forms of *Coccidioides immitis* infection. There were three false-negative reactions by counterimmunoelectrophoresis and one by complement fixation. These are depicted by points below the line marked UD. CSF = cerebrospinal fluid; UD = undiluted.

electrophoresis and double immunodiffusion in this series, but no simultaneous comparisons were done with mycelial antigens. However, this was shown to be the case in the complement fixation method when the spherulin antigen was used in this study and has been reported previously (25).

Complement fixation titers were shown to have an excellent correlation with counterimmunoelectrophoresis titers (Figures 3 and 4). Complement fixation titers of 1:16 to 1:32, which frequently are associated with the disseminated form of the disease (19, 20), were found to correlate with counterimmunoelectrophoresis titers of 1:8 and 1:16. In suspected cases of disseminated infection, antibody titers should be measured over a period of several months because in the acute untreated form of coccidioidomycosis titers may be as high as 1:32, after which they drop rapidly, as was observed in one of our patients.

Counterimmunoelectrophoresis seems to be as reliable a diagnostic tool as the complement fixation test in the serologic follow-up and prognosis of three patients with active coccidioidomycosis infections over periods of 7, 11, and 12 months. Good correlation between both tests was found during the period of time in which the follow-up was carried out. In the patient with meningeal infection the concomitant serologic evaluation of serum and spinal and ventricular fluids was possible. Uniformly, complement fixation and counterimmunoelectrophoresis titers in ventricular fluid were one dilution lower than the titers observed in spinal fluid. This observation has been previously reported (26).

In summary, these data suggest that counterimmunoelectrophoresis is an excellent method for the diagnosis of patients with active coccidioidomycosis infections because of its sensitivity and specificity, the rapidity of reaction, and its good correlation with complement fixation and double immunodiffusion tests. In addition, it may be a useful method for evaluating prognosis and serologic follow-up of patients with the chronic and disseminated form of the disease.

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