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DEVELOPMENT AND EVALUATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF ANTIBODIES TO COCCIDIOIDES IMMITIS

THE UNIVERSITY OF ARIZONA

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DEVELOPMENT AND EVALUATION OF AN
ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION
OF ANTIBODIES TO COCCIDIOIDES IMMITIS

by

Elizabeth Ann Shaffer

A Thesis Submitted to the Faculty of the
DEPARTMENT OF MICROBIOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
MASTER OF SCIENCE
In the Graduate College
THE UNIVERSITY OF ARIZONA

1982
STATEMENT BY AUTHOR

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed as a possible aid in the diagnosis of coccidioidomycosis. This ELISA was used to detect both IgG and IgM antibodies to *Coccidioides immitis* during different stages of illness. A tentative interpretation of the ELISA test was based on a statistical analysis of assays on sera from 57 persons who were either healthy or known to have coccidioidomycosis. The ELISA was compared to latex particle agglutination and complement fixation using 97 sera, and to immunodiffusion using 73 sera, from persons who were either healthy or had symptoms of coccidioidomycosis. The coccidioidin ELISA and the *C. immitis* F antigen ELISA were shown to cross-react with sera from patients known to be infected with *Histoplasma capsulatum* or *Blastomyces dermatitidis*. The coccidioidin ELISA and the latex particle agglutination test appear to be more useful methods for detecting antibodies to *C. immitis* in early primary coccidioidomycosis. The ELISA, because of its ability to discriminate between IgG and IgM antibodies, should prove useful in diagnosing and prognosticating illness.
CHAPTER 1

INTRODUCTION

Coccidioidomycosis is a fungal disease found in the desert around Tucson, the southwestern United States, and northern Mexico. The disease is caused by *Coccidioides immitis*, which can be found growing in the soils of these regions. The inhalation of the arthrospores of the fungus causes a disease of the lower respiratory tract. Most often, the disease is self-limiting and asymptomatic, but coccidioidomycosis can also cause symptoms that are flu-like. In a few cases, the disease is not self-limiting, but spreads to other body organs. This stage of illness is known as secondary (systemic) coccidioidomycosis and is often fatal. About 0.5% of the people who inhale arthrospores of *C. immitis* contract secondary coccidioidomycosis and about 50% of these die.

Diagnosis of the disease is based on clinical signs and symptoms, on serologic tests, and on growth of the organism from patient sputum or lung tissue. The clinical symptoms often mimic symptoms of other respiratory diseases, such as tuberculosis and histoplasmosis. This has caused the clinician to rely heavily on the results of serologic tests. At present, the tests used most often include complement fixation, latex particle agglutination, and double immunodiffusion. The complement fixation test is too difficult for most hospital laboratories to perform and often cross-reacts with other fungal antigens.
Whereas the latex particle agglutination and the double immunodiffusion are much simpler to perform, they have problems with sensitivity and specificity.

The enzyme-linked immunosorbent assay (ELISA) techniques have come into wide use recently to detect antibody concentrations to a variety of bacterial, fungal, viral, and parasitic diseases. The ELISA has been shown to be highly sensitive and capable of a high degree of specificity when used with purified antigens. Results can be obtained within a few hours using a simple spectrophotometer. The reagents for the test are also relatively inexpensive.

There is a need in the clinical laboratory for a diagnostic tool that will combine all the advantages of the complement fixation, latex particle agglutination, and double immunodiffusion tests while minimizing their disadvantages.

The object of this study was to develop an enzyme immunoassay to detect both IgG and IgM antibodies to C. immitis, to compare this ELISA test to tests currently in use, and to test the ELISA developed for cross-reactivity to other fungal antigens.
Coccidioidomycosis

Ecology and Epidemiology

Coccidioidomycosis is a fungal disease of the lower respiratory tract which is caused by Coccidioides immitis. This disease is often asymptomatic but occasionally causes severe or fatal infections in man (85). C. immitis is found growing in the soil of endemic areas which include the southwestern United States (67), northern Mexico (40), Venezuela (17, 18), Columbia (88), Paraguay, Argentina (85), and parts of Central America (71). The disease has also been reported in areas outside the western hemisphere, namely, Nigeria, Pakistan, central Asia, and Russia, but it is unclear as to whether these cases are due to the organism actually growing in the soils of these countries or these infections were contracted while in an endemic area (85). In the United States, the fungus is found in Arizona, Nevada, New Mexico, Utah, Texas, and southern California, particularly the San Joaquin Valley (60, 62, 63, 64, 65, 66, 67).

C. immitis survives in the lower Sonoran life zones where there is a hot dry season followed by a wet season. Endemic soil, which usually has a high concentration of salt and organic matter (28, 29), becomes sterile during the hot dry season and then is reinvaded by the
fungus after the moisture of the rainy season causes increased salt content. The infectious form of the organism is the arthrospore, which is disseminated by dust aerosols that occur in late summer and are then inhaled by animals and humans. Coccidioidomycosis has been seen in various animals indigenous to this life zone (horses, cattle, swine, llamas, burrows, rabbits, sheep, and rodents (6)), with the systemic form of the disease occurring in dogs (61, 107), coyotes (106), and primates (72). Transmission of the disease is by dust aerosols and does not appear to be transmitted man to man (58).

Clinical Aspects

The first reported case of coccidioidomycosis occurred in an Argentine soldier in 1892 (84), and was at first believed to be a neoplasm (mucosis fungoides) with an unidentified parasite within the lesion. The organism resembled the protozoan of the order Coccidia, and hence was named Coccidioides immitis (87). Posadas (84) published his preliminary results of the Argentine case in 1892 (124).

Subsequently, a Portuguese laborer who had migrated to the San Joaquin Valley became ill with a disease similar to the case reported by Posadas (84). Rixford and Gilchrist (87) studied the parasite and in 1896 published an account of both cases, recognizing coccidioidomycosis as a new infectious disease. Ophulus and Moffitt (79) described the etiologic agent of the disease as being a fungus and, in 1905, Ophulus described the life cycle of the fungus (78). During the following decade, it became apparent that the disease had a respiratory route of infection, instead of infecting through the skin. Dickson and
Gifford in 1938 (26) reported on the occurrence of a mild or self-limiting form of the disease and its relationship to more severe forms.

Today, there are two forms of the disease that are recognized, primary coccidioidomycosis and secondary coccidioidomycosis. Mild infection (primary coccidioidomycosis) results from inhalation of dust which contains the arthrospore. This infection can be completely asymptomatic or produce a mildly severe pulmonary or lymphatic lesion. Clinical symptoms include fever, malaise, slight dry cough, and chest pain, with occasional symptoms of night sweats and anorexia. A small percentage of individuals who become infected show clinical symptoms of disease. Whether clinical symptoms occur or not is related to the number of arthrospores inhaled and to the immune status of the host (127). Asymptomatic patients are known as "skin test convertors"; they become reactive to intradermal injections of coccidioidin in the absense of demonstrable disease.

Approximately 5%-10% of the patients contracting coccidioidomycosis develop residual cavitary lesions, which are thin-walled lesions in the lungs and lymph nodes that usually heal spontaneously. Erythema nodosum is also a common clinical symptom in the primary form of the disease, and such appearance coincides with the development of delayed hypersensitivity to fungal proteins.

Secondary coccidioidomycosis (49) is blood or lymph borne and the lesions tend to be suppurative as opposed to the granulomatous lesions in primary disease. Disseminated disease usually occurs in the immunologically comprised host or in patients who may have other
underlying diseases. This form can involve any tissue or organ and is characterized by the verrucous granulomas which occur as dermal or internal lesions. These lesions can either heal, or they can persist and spread until the host dies (37).

Some individuals respond immunologically to the disease while others do not. In people who do respond, dissemination does not occur unless there is a breakdown in immunity caused by old age, cancer, or other predisposing factors. In people who do not respond, dissemination occurs early in the progress of the disease. Race and sex appear to be determining factors in people who do not respond immunologically to the disease. Men have a dissemination rate four to six times as great as women (35), except during pregnancy when steroid levels (27) significantly increase the incidence of dissemination in females (43, 97, 98, 112). Phillipine males seem to have a dissemination rate 50 times that of white males, and black males have a dissemination rate ten times that of white males (127). These results are in question, though, because the studies were done in areas where there was a higher population of black and Phillipine males than white males.

Upon inhalation into the respiratory tract, the arthrospore develops into a spherule containing many endospores. The cellular reaction to the spherule is a proliferation of mononuclear cells with giant cell formation. This reaction is frequently called a tuberculoid lesion and is a characteristic feature of the cell-mediated immune response as seen in many infectious diseases, such as tuberculosis,
leprosy, syphilis, typhoid fever, tularemia, brucellosis, sarcoidosis, sporotrichosis, histoplasmosis, and blastomycosis (36).

Diagnosis

Diagnosis of the disease at the present time is based on growth of the organism from tissue and serologic tests such as the skin test, the complement fixation test, and the precipitin tests.

Delayed hypersensitivity to fungal proteins of *C. immitis* develops in 80% of patients having the primary disease within the first week of illness; and, by the second week, 90% of patients have developed this hypersensitivity (34). This delayed hypersensitivity is the basis of the reaction to the intradermal skin test. The standard procedure for the use of the coccidioidin antigen in the test was developed by Smith in 1948 (104). A positive skin test, an induration of at least 5 mm, is only indicative of past exposure to disease and not necessarily an indication of present infection. Furthermore, the skin test occasionally becomes negative during disseminated disease due to an overwhelming of the immune system with fungal antigens. It has also been shown that delayed hypersensitivity to other fungal antigens will cross-react with coccidioidin to cause a false positive reaction (23, 34, 99, 102, 128).

Precipitin tests, such as latex agglutination (C. Mulder, S. Kiddy, and K. Lou, Bacteriol. Proc. 1966, p. 141), and immunodiffusion (50, 51, 52, 55), primarily detect the IgM class (83, 93) of antibodies which appears early in the course of primary coccidioidomycosis. By the second week of illness, 80% of the sera from coccidioidomycosis
patients develop these antibodies (82, 100), but they do not persist
and by the second month of illness these antibodies begin to disappear.
These antibodies may actually be at levels so low that they cannot be
detected by the above methods. Occasionally patients with disseminated
disease will have a persistence of the IgM antibody (82). Both
latex agglutination and immunodiffusion tests are simple to perform and
the results are reproducible (51), but false negative results can be
obtained by the titer declining to insignificant levels long after
initial infection.

The complement fixation test (101, 103) detects the IgG class
of antibodies which appears late in the course of primary coccidioido-
mycosis. By the second month of illness, over 50% of the patients show
positive titers (82, 100), and by the seventh month, 90% of the pa-
tients have become positive (103). These antibodies may persist in
low titers for years after resolution of the primary infection.
Titers to these antibodies rise proportionally to the severity of
disease and may be used as a prognostic tool (101). This test is
often difficult for diagnostic laboratories to perform routinely and
must be sent to a reference laboratory. Strict adherence to method-
ology for this test must be observed to obtain reproducible results
(103, 122). Sera from patients with coccidioidomycosis may appear
negative during the first weeks of illness, giving limited value to
this test in providing an early diagnosis of the disease.
Enzyme-Linked Immunosorbent Assay

Procedures for conjugating immunoglobulins to other substances have been known for a long time and are widely used. However, the technique known as enzyme-linked immunosorbent assay (ELISA) was not developed until 1967 when Nakane and Pierce (76) devised a method for conjugating an enzyme to an immunoglobulin. Also, the development of a method, by Engvall (30, 31, 74) and Van Weeman (109, 110), to adsorb an antigen or antibody to a solid phase such as polystyrene provided the technical basis of the present day solid phase ELISA techniques.

The solid phase of the ELISA procedure, which must show reproducible uptake of protein by passive adsorption in alkaline (pH 9.6) conditions (116, 118), can be made of silicone rubber, micro-crystalline glass, polyacrylamide, or plastics, especially polystyrene. There is an advantage in using polystyrene microtiter plates in that during the washing procedure, there is no need for centrifugation. Plates can be coated overnight at 4°C, dried the next morning, and sealed. This procedure increases the shelf life to a year or more (117). Due to the high affinity of protein for polystyrene, it is necessary to dilute test sera in buffers which contain wetting agents, such as Tween-20.

A variety of enzymes may be conjugated to the antibody. These include cytochrome C, glucose oxidase, lactic dehydrogenase, alkaline phosphatase, and horse radish peroxidase. The two most popular seem to be alkaline phosphatase and horse radish peroxidase. The choice of an enzyme is made on the basis of low cost, ease of conjugation, stability, high reactivity, and safety. There are two methods for conjugating the
enzyme to the protein. One method uses sodium periodate (75), but the
most popular method uses glutaraldehyde in a one-step or a two-step
procedure (7, 8). The two-step method yields more homogenous conjugates in that only one molecule of enzyme is linked to one molecule of antibody. Conjugates prepared in this manner are stable and can be used by diluting them in phosphate-buffered saline with Tween-20.

Substrates for enzymes must permit the detection of very small amounts of conjugate. Substrates which are initially colorless and yield a colored product upon hydrolysis are most frequently used. One of the best substrates for alkaline phosphatase is p-nitrophenylphosphate (117). The reaction can be stopped with 3 M NaOH and read spectrophotometrically at 405 nm. The best substrate for peroxidase is o-phenylenediamine (117). This reaction can be stopped by adding H$_2$SO$_4$ and is read spectrophotometrically at 429 nm.

A variety of modifications of the basic ELISA technique are now in widespread use; generally, these techniques involve a solid-phase antigen or antibody and may be used to measure antigens or antibodies in test fluids.

Double-Antibody Sandwich Technique

In the double-antibody sandwich technique (Figure 1), antibody is adsorbed onto the polystyrene surface. A test solution thought to contain antigen is added and allowed to react with the antibody. This forms a stable antibody-antigen complex which is attached to the solid phase. The next step involves the addition of an enzyme-labelled antibody specific for the test antigen. This forms an antibody-
1 Antibody adsorbed to plate

wash

2 Test solution containing antigen added

wash

3 Add enzyme labelled specific antibody

wash

4 Add enzyme substrate

Figure 1. The Double Antibody Sandwich ELISA for Measuring Antigen
antigen-enzyme-labelled-antibody complex attached to the solid phase. Up to this point, the interaction of reagents is not visible to the naked eye. Visualization of the whole complex is accomplished by a colorimetric assay for enzyme activity. A colorless substrate is hydrolyzed to produce a colored product that is read photometrically (116).

This technique has been used to assay hormones (13, 108, 109, 110, 111), serum components (9, 12), immune complexes (3), rheumatoid factor (68, 125), bacterial antigens (11, 105, 126), fungal antigens (124), and a variety of antigens important in veterinary and agricultural sciences (2, 94, 119).

Competitive Technique

In the competitive technique (Figure 2), antibody is adsorbed onto the polystyrene surface. This procedure requires two wells; the first well is incubated with enzyme-labelled antigen alone, and the second well is incubated with enzyme-labelled antigen plus a test solution containing an unknown amount of antigen. The first well serves as a reference well. This step in the reference well results in stable antibody-enzyme-labelled-antigen complexes. The second well incubated with both enzyme-labelled antigen and unknown antigen serves as the test well. This step results in stable antibody-antigen complexes, some of which are actually antibody-enzyme-labelled-antigen complexes. The next step is to add substrate to both the reference and test wells. Enzyme in the reference well will result in hydrolysis of the substrate to produce a colored product. In the test well,
Figure 2. The Competitive Method of ELISA for Assaying Antigen
color production can only be due to labelled antigen and the difference in the intensity of color between the wells is the result of unknown antigen competitively binding to the antibody (116).

This technique has been useful in detecting drugs (4, 70, 80), hormones (5), different serum components (39), oncofoetal protein (10, 22, 24, 42), aflatoxin B antigen (59), staphylococcal enterotoxin (11, 105), and fungal kojic acid antigen (1).

Indirect Technique

The indirect ELISA technique (Figure 3) is performed with antigen adsorbed to the polystyrene surface. A test solution thought to contain antibody is added to the well and allowed to react with the antigen. If antibody is present, a stable antigen-antibody complex is formed. The next step is the addition of the enzyme-labelled-anti-immunoglobulin. This reacts to form an antigen-antibody-enzyme-labelled-antibody complex in the well. Visualization of this complex is accomplished by adding substrate, followed by colorimetric quantitation of the products.

This technique has been found useful in detecting antibodies to a variety of infectious agents, such as Salmonella (20, 21, 96), Brucella (19, 44), Yersinia (19, 41), Klebsiella (86), Legionella (32), Streptococcus (90), Neisseria (38), Vibrio (45, 46, 92), Treponema (113), Mycobacterium (77), Aspergillus (47), Cryptococcus (95), Nocardia (73), dermatophytes (56), Candida (57, 123), viral agents (54, 69, 114, 115, 118), and parasites (48, 89, 120, 121).
1 Antigen adsorbed
to plate

wash

2 Add serum: any
specific antibody
attaches to antigen

wash

3 Add enzyme labelled
antiglobulin which
attaches to antibody

wash

4 Add substrate

Figure 3. The Indirect Method for Assay of Antibody
CHAPTER 3

MATERIALS AND METHODS

Patient Sera

Positive patient sera were obtained from clinics in the Tucson area. Sera were collected both from healthy persons and from individuals having clinical or serological evidence of infection with Coccidioides immitis. Tests used to establish the diagnosis included complement fixation, immunodiffusion, and latex agglutination. In addition, sera were obtained from 19 healthy donors (medical students) in Gainesville, Florida. These donors had no previous history of exposure to areas endemic for C. immitis and were skin test negative. Human reference sera with antibodies to either Histoplasma capsulatum or Blastomyces dermatitidis were obtained from the Center for Disease Control, Atlanta, Georgia.

Immunochemical Reagents and Chemicals

Anti-human IgG and IgM-alkaline phosphatase conjugates were obtained from Sigma Chemical Company (A-3150; A-3275). Conjugates were diluted 1:10 upon receipt, divided into portions, and frozen until needed. Immediately prior to use, the conjugates were further diluted to the appropriate working dilution. The substrate, p-nitrophenylphosphate, was purchased in tablet form from Sigma Chemical Company (104-105). Tablets were stored at -20 C until needed.
Immediately prior to use, one tablet was dissolved in 5 ml of diethanolamine buffer, pH 9.8 (10% w/v). Coccidioidin was purchased from MA Bioproducts (50-323J). All chemicals used were ACS grade, unless otherwise specified.

Coating of Antigen to Wells

An indirect ELISA technique (116), Figure 3, was used. Coccidioidin (MA Bioproducts) was adsorbed to the wells of microtiter plates (Dynatech, U-bottom, Immulon I) by adding 300 ul of various dilutions of coccidioidin in coating buffer (50 mM bicarbonate buffer, pH 9.6; 0.02% Sodium Azide) and incubating the plates overnight at 4 C. After incubation, the plates were rinsed once with PBS-Tween (10 mM KPO₄, pH 7.2; 0.15 M NaCl; 2 mM KCl; 0.05% Tween 20; 0.02% Sodium Azide), once with distilled water, dried with a vacuum pump, and sealed with tape for storage. Plates were stored at 4 C until needed. Immediately prior to performing the ELISA, the wells of the microtiter plates were washed once again with PBS-Tween.

ELISA Procedure

To perform the assay, 200 ul of diluted patient serum was added to each sample well (in triplicate) and incubated for one hour at room temperature with vigorous agitation on a Dynatech Micro-Shaker II. After incubation, the sample wells were rinsed three times with PBS-Tween, then 200 ul of anti-human globulin-enzyme conjugate was added to each sample well and incubated with agitation for one hour at room temperature. Following incubation with conjugate, the sample wells
were again rinsed three times with PBS-Tween and 200 ul of substrate (p-nitrophenyl phosphate, 1 mg per ml in 10% (w/v) diethanolamine, pH 9.8) was added to each well and incubated with agitation at room temperature for 30 minutes. After incubation with substrate, the reaction was stopped by adding 50 ul of 3 M NaOH to each well. The contents of the sample well were then diluted into a tube containing 500 ul of a diluting buffer (3 parts diethanolamine + 1 part 3 M NaOH) and mixed thoroughly. The optical density of this solution was read on a Gilford Micro-Sample Spectrophotometer at 405 nm.

**Interpretation of Results**

The optical density values obtained from the ELISA procedure were evaluated by first subtracting the optical density of the reagent blank from the optical densities of the test sera and the positive and negative control sera. The optical densities of test sera were then divided by the optical density of a reference negative serum to obtain a ratio of the optical density of the test serum to that of the negative serum. This ratio was referred to as the positive to negative ratio, or simply P/N. The P/N value was determined in triplicate for each serum tested. For purposes of statistical analysis, the P/N values were converted to logarithms.

**Other Serological Tests**

Sera were tested for antibodies to *C. immitis* using an immunodiffusion test kit for coccidioidomycosis purchased from Nolan Biological Laboratories, Atlanta, Georgia. Tests were performed and
interpreted according to the manufacturer's recommendations. Latex particle agglutination tests were performed using the Coccidioides Latex Agglutination System purchased from Meridian Diagnostics, Inc., Cincinnati, Ohio. Tests were performed and interpreted according to the manufacturer's recommendations. The complement fixation test was performed as described previously (81) using a microtiter technique.
CHAPTER 4

RESULTS

Development of the ELISA

A checkerboard titration was performed on patient sera, rabbit anti-human immunoglobulin-alkaline phosphatase conjugate and coccidioidin to determine their optimum dilutions. Optimum dilutions were those that produced the highest P/N value for each of three known positive sera in different stage of illness. The results are shown in Figures 4, 5, 6, and 7. A 1:40 and 1:80 dilution of patient sera was optimal for the IgG and IgM ELISA, respectively. A 1:100 dilution of coccidioidin and a 1:1000 dilution of conjugate was the best choice for all three patients for both IgG and IgM ELISA. The day-to-day variation of P/N ratios was analyzed (Appendix A, Tables 11, 12).

Statistical Analysis

In order to define the range of positive and negative ELISA P/N values, IgG and IgM ELISA were performed on 19 sera from patients with other serological and clinical evidence of infection with C. immitis. These 19 sera were designated as positive. In addition, the ELISA was also performed on two other groups of 19 sera. One group, the negative Tucson group, consisted of individuals living in the Tucson, Arizona, area and having no serological or clinical evidence of infection. The other group, the negative Florida group, consisted of
Figure 4. Log_{10} P/N Values for a Checkerboard Titration with Sera from Patient A, Using 3 Dilutions of Sera, 3 Concentrations of Antigen, and 3 Dilutions of IgG Conjugate

See Appendix A, Table 6, for raw data.
<table>
<thead>
<tr>
<th>Dilution</th>
<th>1:50</th>
<th>1:100</th>
<th>1:200</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANTIGEN</td>
<td>☐</td>
<td>○</td>
<td>□</td>
</tr>
<tr>
<td>CONJUGATE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERUM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4. \( \log_{10} \) P/N Values - Sera from Patient A
Figure 5. Log₁₀ P/N Values for a Checkerboard Titration with Sera from Patient B, Using 3 Dilutions of Sera, 3 Concentrations of Antigen, and 3 Dilutions of IgG Conjugate

See Appendix A, Table 7, for raw data.
Figure 6. Log10 P/N Values for a Checkerboard Titration with Sera from Patient C, Using 3 Dilutions of Sera, 3 Concentrations of Antigen, and 3 Dilutions of IgG Conjugate

See Appendix A, Table 8, for raw data.
Figure 7. Log_{10} P/N Values for a Checkerboard Titration with Sera from Patient C, Using 5 Dilutions of Sera, 3 Concentrations of Antigen, and 3 Dilutions of IgM Conjugate

See Appendix A, Table 9, for raw data.
medical students living in the Gainesville, Florida, area and having no serological or clinical evidence of systemic fungal infection and no history of exposure to regions endemic for *C. immitis*, *B. dermatitidis*, or *H. capsulatum*. The latter group was included to compare the effects of living in an endemic area on the P/N value of persons thought to have had exposure to *C. immitis*.

The individual IgG and IgM P/N values from the above groups were converted to their corresponding Log_{10} values and analyzed using a one-way analysis of variance. There was no significant difference between the means of the negative Tucson group and the negative Florida group. Therefore, these groups were pooled and treated as one negative group (n = 38). The calculated means and standard errors were used to construct curves of the normal distributions for IgG and IgM P/N values of all groups (Figure 8). The confidence limits were calculated at the 0.05 probability level.

For serologically negative sera, 89% of the population will have an IgG Log_{10} P/N value of ≤0.848, and 2.5% of the population will have an IgG Log_{10} P/N value of ≥1.063 and be erroneously classified as positive. The remaining 8.5% of the population would be classified as doubtful. Seventy-eight percent of the population will have an IgM Log_{10} P/N value of ≤0.834, and 2.5% of the population will have an IgM Log_{10} P/N value of ≥1.297 and be erroneously classified as positive. The remaining 19.5% of the population would be classified as doubtful.

For serologically positive sera, 89% of the population will have an IgG Log_{10} P/N value of ≥1.063, and 2.5% of the population
Figure 8. Normal Distributions for Log_{10} of IgG and IgM P/N Values for Positive and Negative Sera

See Appendix A, Table 10, for raw data.
will have an IgG \( \log_{10} \) P/N value of \( \leq 0.848 \) and be erroneously classified as negative. The remaining 8.5% of the population would be classified as doubtful. Seventy-eight percent of the population will have an IgM \( \log_{10} \) P/N value of \( \geq 1.297 \), and 2.5% of the population will have an IgM \( \log_{10} \) P/N value of \( \leq 0.834 \) and be erroneously classified as negative. The remaining 19.5% of the population would be classified as doubtful. The higher percentage of the population classified as doubtful by the IgM ELISA, as compared to the IgG ELISA, is due to a greater standard error for the IgM ELISA.

Comparison of ELISA with Other Serological Tests

A total of 97 sera was tested by IgG and IgM ELISA, LPA, and CF tests (Table 1). Seventy-three of these sera were also tested by the DID test (Table 2). ELISA results were interpreted as either positive, negative, or doubtful according to the distributions described earlier. To be considered negative, a serum must be negative by both IgG and IgM ELISA. To be considered doubtful, a serum must be classified as either IgG or IgM doubtful, or both. To be considered positive by ELISA, a serum must be classified as either IgG or IgM positive, or both. CF tests were interpreted as positive, negative, or doubtful. CF titers \( \geq 1:8 \) were considered positive, titers of 1:4 and 1:2 were considered doubtful, and titers \( \leq 1:2 \) were considered negative. LPA and DID tests were interpreted as either positive or negative.

The LPA test gave a higher percentage of positive sera (90.7%), followed by the IgG + IgM ELISA (62.8%), the CF (11.3%), and DID (5.5%).
Table 1. Comparison of *Coccidioides* ELISA with LPA and CF Tests

<table>
<thead>
<tr>
<th>ELISA</th>
<th>IgG</th>
<th>IgM</th>
<th>IgG + IgM</th>
<th>LPA</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>41/97(42.3%)</td>
<td>45/97(46.4%)</td>
<td>61/97(62.9%)</td>
<td>88/97(90.7%)</td>
<td>11/97(11.3%)</td>
</tr>
<tr>
<td>Doubtful</td>
<td>21/97(21.6%)</td>
<td>34/97(35.1%)</td>
<td>26/97(26.8%)</td>
<td>2/97(2.1%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>35/97(36.1%)</td>
<td>18/97(18.5%)</td>
<td>10/97(10.3%)</td>
<td>9/97(9.3%)</td>
<td>84/97(86.6%)</td>
</tr>
</tbody>
</table>
Table 2. Comparison of *Coccidioides* ELISA with LPA, CF, and DID Tests

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th>LPA</th>
<th>CF</th>
<th>DID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
<td>IgG + IgM</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>33/73(45.2%)</td>
<td>35/73(48.0%)</td>
<td>49/73(67.1%)</td>
<td>64/73(87.7%)</td>
</tr>
<tr>
<td>Doubtful</td>
<td>17/73(23.3%)</td>
<td>26/73(35.6%)</td>
<td>18/73(24.7%)</td>
<td>NA²</td>
</tr>
<tr>
<td>Negative</td>
<td>23/73(31.5%)</td>
<td>12/73(16.4%)</td>
<td>6/73(8.2%)</td>
<td>9/73(12.3%)</td>
</tr>
</tbody>
</table>

1Positive sera were IgG or IgM ELISA positive; negative sera were IgG and IgM negative.

²Not applicable.
The combined IgG and IgM ELISA was more effective in the recognition of positive sera than the IgG or IgM alone. Of those sera with a positive IgG ELISA, 29% had a negative or doubtful IgM. Of those sera with a positive IgM ELISA, however, 42% had a negative or doubtful IgG ELISA.

The data were analyzed by intercorrelation analysis (Table 3). At the p = 0.01 level, the CF showed a significant correlation to both IgG ELISA and the sum of the IgG and IgM ELISA. The CF showed no significant correlation with the IgM ELISA. The LPA test showed no significant correlation with the IgG or IgM ELISA or with the sum of the IgG and IgM ELISA. According to Table 3, the correlation coefficient (r) equalled .3442, which means that $r^2 = .12$, or 12% of the variation found in the CF results can be explained by the variation in the IgG ELISA results.

Since the CF test and the IgG ELISA showed the greatest correlation, the same 97 sera were analyzed by bivariate analysis. The $Y_1$ axis is $\log_{10}$ IgG ELISA P/N, and the $Y_2$ axis is CF titer. The equation of the principle axis was $Y_1 = .94499 + .09350Y_2$, and the equation of the minor axis was $Y_1 = 6.83976 - 10.69505Y_2$. The 95% confidence limits of the slope of the principle axis are $L_1 = 0.4293$ and $L_2 = 0.14455$.

IgG and IgM Antibodies During Different Stages of Illness

A patient known to have primary disease was evaluated with the IgG and IgM ELISA for one year. The results are shown in Figure 9. The first serum sample was drawn and an X-ray was taken at the onset of symptoms. The chest X-ray showed a coin lesion in the lung, and the serum sample was in the positive range for IgM, negative range for
Table 3. Intercorrelation Analysis of 97 Sera Between CF and Coccidioidin ELISA

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>IgM</th>
<th>IgG + IgM</th>
<th>LPA</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>1.0000</td>
<td>0.4067</td>
<td>0.8134</td>
<td>0.1558</td>
<td>0.1442</td>
</tr>
<tr>
<td>IgM</td>
<td>1.0000</td>
<td>0.8622</td>
<td>0.1582</td>
<td>0.1665</td>
<td></td>
</tr>
<tr>
<td>IgG + IgM</td>
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<td></td>
<td>0.1880</td>
<td>0.2969</td>
<td></td>
</tr>
<tr>
<td>LPA</td>
<td>1.0000</td>
<td></td>
<td></td>
<td>-0.0296</td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0000</td>
</tr>
</tbody>
</table>

Critical Value = 0.267 at p = 0.01.
Figure 9. Log_{10} IgG and IgM P/N Values of a Patient with Primary Coccidioidomycosis
IgG, LPA positive, DID negative, and had a CF titer of 1:16. The last serum sample was taken while the patient was still experiencing symptoms of disease a year after the initial onset of illness. The IgM was in the doubtful range, the IgG was in the positive range, LPA was negative, DID was negative, and the CF titer was negative.

Figure 10 shows another comparison of the IgG and IgM ELISA. Each patient represents a different clinical stage of infection. Patient A had disseminated disease with underlying sarcoidosis for more than one year. Patient B had early primary infection. Patient C had primary infection. Patient D had an asymptomatic infection (skin-test convertor) five years previously. Patient E had an asymptomatic infection. Patient F was not known to have been exposed to any systemic fungal agent indigenous to the United States.

Cross-Reactivity of the ELISA with Histoplasmosis and Blastomycosis Positive Sera

The cross-reactivity of the coccidioidin ELISA to antibodies against other systemic fungal infections was tested by assaying high and low titer sera from patients known to be infected with *H. capsulatum* or *B. dermatitidis* (Table 4). The histoplasmosis sera \( (n = 5) \) gave \( \log_{10} \) IgG ELISA P/N values ranging from 1.126 to 1.719 \( (\bar{x} = 1.396) \), and \( \log_{10} \) IgM ELISA P/N values ranging from 0.649 to 1.331 \( (\bar{x} = 1.087) \). The blastomycosis sera \( (n = 5) \) gave \( \log_{10} \) IgG P/N values ranging from 0.389 to 1.374 \( (\bar{x} = 0.890) \), and \( \log_{10} \) IgM P/N values ranging from 1.177 to 1.650 \( (\bar{x} = 1.348) \). All five histoplasmosis sera and four of five
Figure 10. Log$_{10}$ IgG and IgM P/N Values for 5 Patients in Different Stages of Illness
Table 4. Cross-Reactivity of Coccidioidin ELISA with Sera from Patients with Histoplasmosis or Blastomycosis

<table>
<thead>
<tr>
<th>Serum</th>
<th>IgG</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histoplasmosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7935</td>
<td>52.9</td>
<td>5.8</td>
</tr>
<tr>
<td>9155</td>
<td>16.9</td>
<td>21.5</td>
</tr>
<tr>
<td>10716</td>
<td>21.6</td>
<td>16.8</td>
</tr>
<tr>
<td>20807</td>
<td>38.2</td>
<td>30.1</td>
</tr>
<tr>
<td>20808</td>
<td>13.6</td>
<td>30.1</td>
</tr>
<tr>
<td>Blastomycosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7936</td>
<td>23.8</td>
<td>13.4</td>
</tr>
<tr>
<td>7937</td>
<td>8.4</td>
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<td>8355</td>
<td>12.5</td>
<td>23.5</td>
</tr>
<tr>
<td>11488</td>
<td>5.1</td>
<td>33.1</td>
</tr>
<tr>
<td>19295</td>
<td>2.5</td>
<td>44.7</td>
</tr>
</tbody>
</table>
blastomycosis sera would be classified as positive by the coccidioidin ELISA, and are therefore cross-reactive with the coccidioidin used in this procedure.

A crude preparation of C. immitis F antigen was also used to test the cross-reactivity of the ELISA procedure (Table 5). The histoplasmosis sera (n = 5) gave Log$_{10}$ IgG P/N values ranging from 0.5760 to 1.2245 ($\bar{x} = 0.8959$). The blastomycosis sera (n = 4) gave Log$_{10}$ IgG P/N values ranging from 0.3291 to 1.1771 ($\bar{x} = 1.0005$). One of five histoplasmosis sera and two blastomycosis sera would be classified as positive, two histoplasmosis sera and two blastomycosis sera would be classified as negative. Therefore, these fungal antibodies are also cross-reactive with the crude C. immitis F antigen used in this procedure.
Table 5. Cross-Reactivity of *C. immitis* F Antigen ELISA with Sera from Patients with Histoplasmosis or Blastomycosis

<table>
<thead>
<tr>
<th>Serum</th>
<th>IgG</th>
<th>Histoplasmosis</th>
<th>Blastomycosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7935</td>
<td>7936</td>
</tr>
<tr>
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<td></td>
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<td>10716</td>
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<td></td>
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<td>20807</td>
<td>11488</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20808</td>
<td></td>
</tr>
</tbody>
</table>

Histoplasmosis

- Serum 7935: IgG 1.0427
- Serum 9155: IgG 0.9542
- Serum 10716: IgG 0.6564
- Serum 20807: IgG 1.2245
- Serum 20808: IgG 0.5760

$\bar{x} = 0.8908$

Blastomycosis

- Serum 7936: IgG 1.1771
- Serum 7937: IgG 0.3291
- Serum 8355: IgG 1.0212
- Serum 11488: IgG 0.7947

$\bar{x} = 0.8305$
The diagnosis of coccidioidomycosis is made difficult since the symptoms and clinical findings mimic other respiratory diseases. The serologic tests used for diagnosing disease do not always detect low concentrations of antibodies that occur during the onset of primary disease. These tests may also show a degree of non-specificity. Since ELISA procedures are known to be sensitive and capable of a high degree of specificity, an ELISA procedure for the detection of antibodies to \textit{C. immitis} was developed in this study.

When the ELISA test was in the first stages of development, the day-to-day variation of the optical densities of any given serum complicated attempts to standardize the procedure. Researchers in the past have dealt with this problem in different ways. One way the photometrically-derived results can be expressed is as an end point titer. In this method, the sera are serially diluted and all dilutions are tested by the ELISA. The titer is the last dilution which yields a value above that of a group of known negative samples (69). A second way the results can be expressed is the multiple of normal activity (MONA) of negative sera. This method is based on the dose response curve of each system, which enables values more representative of the serum titer to be obtained from tests on a single serum dilution (33). The method used in this study is based on a ratio between positive and
negative sera (25). Previous investigators have considered two to three times the negative value as a positive ELISA result. It should be remembered, though, that in antibody assays the end result reflects the combined effects of antibody concentration and its affinity for the antigen (14, 15).

The coccidioidin ELISA developed in this study was evaluated by comparing it to the CF test and to two commercial test kits, the DID and LPA tests. These tests were performed on sera collected from healthy donors, patients suffering from disseminated coccidioidomycosis, and persons suspected of having primary coccidioidomycosis. A majority of these sera were obtained from private out-patient clinics, and generally a diagnosis with follow-up examinations was not available. It is likely that many of the patients from whom these sera were obtained were in early stages of primary infection. This speculation is supported by the observation that 42% of the patients with a positive IgM ELISA had a negative or doubtful IgG ELISA.

The ELISA and the LPA showed a positive result more often than the DID or CF test. These results are not surprising, since the CF and the C. immitis F antigen DID tests are known (51) to detect primary antibodies that appear later in infection, and most of the sera that were tested were within the first month of illness. Because of this, the CF and the F antigen DID are of limited value as an aid in diagnosing early primary coccidioidomycosis. These tests have been shown previously to be valuable in diagnosing and prognosticating later stages
of illness (51, 103). In this study, all CF and DID test positive sera were also positive by the ELISA.

The LPA test appeared to be more sensitive than the ELISA. However, a previous study (53) showed that the LPA test has a small percentage (6%) of false positive results. The manufacturer of the LPA test kit used in this study reports that a false positive rate of up to 10% may occur. A determination of the actual false positive rate of the test used in this study was not possible since a diagnosis based on clinical findings and follow-up data was not available. In spite of these difficulties, it can be concluded that the ELISA compares to the LPA in screening patients with early primary coccidioidomycosis.

The coccidioidin ELISA was also evaluated by comparing the IgM P/N and the IgG P/N of five patients who had differing stages of illness. These results were not surprising, as it had been previously shown that the IgM antibodies appear first and begin to disappear around the fifth month, while the IgG antibodies begin appearing around the second to third month of illness (103). Further study involving more sera from patients would need to be evaluated to determine if there is a pattern to the positive, doubtful, negative range of ELISA P/N correlating to stage of illness. The five patients seen in Figure 10 suggest that this might be true.

The coccidioidin ELISA and F antigen ELISA showed a high degree of cross-reactivity with sera from patients suffering from histoplasmosis or blastomycosis. Immunologic cross-reactions between these three fungal diseases are well known (16, 91, 102), and the F antigen and
coccidioidin ELISAs in this study do not overcome this problem. There is a need for further studies to identify antigens that are unique to each of these three fungi, so that sensitive and specific serologic tests can be developed. Until such tests are available, the clinical diagnosis of coccidioidomycosis will continue to rely on knowledge of the patient's past exposure to endemic areas.

In conclusion, an ELISA was developed in this study for detecting antibodies to *Coccidioides immitis*. The IgM and IgG P/N ratios obtained correlated well with previous expected results. However, further study needs to be done to find a specific antigen for *C. immitis*. Further study also needs to be done to correlate stage of illness with ELISA P/N values.
APPENDIX A

RAW DATA
Table 6. Raw Data for Figure 4, Patient A

<table>
<thead>
<tr>
<th>Patient Sera</th>
<th>1:50 Ag</th>
<th>1:100 Ag</th>
<th>1:200 Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgG</td>
<td>IgG</td>
</tr>
<tr>
<td>1:500</td>
<td>1.2822</td>
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Table 7. Raw Data for Figure 5, Patient B

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</tr>
</thead>
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Table 8. Raw Data for Figure 6, Patient C

<table>
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<th>1:200 Ag</th>
</tr>
</thead>
<tbody>
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<td>IgG</td>
<td>IgG</td>
<td>IgG</td>
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<td>1:10</td>
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<td>1:20</td>
<td>0.9912</td>
<td>1.1973</td>
<td>1.0774</td>
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<td>1:40</td>
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<td>1.1629</td>
<td>1.0233</td>
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Table 9. Raw Data for Figure 7

<table>
<thead>
<tr>
<th>Patient Sera</th>
<th>1:50 Ag</th>
<th></th>
<th></th>
<th>1:100 Ag</th>
<th></th>
<th></th>
<th>1:200 Ag</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>1:500</td>
<td>1:1000</td>
<td>1:1500</td>
<td>1:500</td>
<td>1:1000</td>
<td>1:1500</td>
<td>1:500</td>
<td>1:1000</td>
<td>1:1500</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>IgM</td>
<td>IgM</td>
<td>IgM</td>
<td>IgM</td>
<td>IgM</td>
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<td>IgM</td>
<td>IgM</td>
</tr>
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<td>0.3010</td>
<td>0.9777</td>
<td>0.7443</td>
<td>0.7672</td>
<td>0.7559</td>
<td>0.6812</td>
<td>0.7924</td>
<td>0.8195</td>
</tr>
<tr>
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<td>0.7993</td>
<td>0.9518</td>
<td>0.9269</td>
<td>0.7324</td>
<td>0.6857</td>
<td>0.6990</td>
<td>0.7482</td>
<td>0.7324</td>
<td>0.5052</td>
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<tr>
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<td>1.0645</td>
<td>1.0212</td>
<td>0.9708</td>
<td>1.0473</td>
<td>0.9138</td>
<td>0.7404</td>
<td>0.8228</td>
<td>0.8388</td>
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<td>1:80</td>
<td>1.055</td>
<td>1.0128</td>
<td>1.1335</td>
<td>0.7559</td>
<td>0.4698</td>
<td>0.4232</td>
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<tr>
<td>1:160</td>
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<td>1.2443</td>
<td>1.2253</td>
<td>1.2135</td>
<td>1.2480</td>
<td>1.1492</td>
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</table>
Table 10. Raw Data for Figure 8

<table>
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<tr>
<th>IgG Log_{10} P/N</th>
<th>IgM Log_{10} P/N</th>
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<tbody>
<tr>
<td>Positive Tucson</td>
<td>Negative Tucson</td>
</tr>
<tr>
<td>Positive Florida</td>
<td>Negative Florida</td>
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Table 11. Summary of Day-to-Day Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>M.S.</th>
<th>F</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum A, B, C</td>
<td>3.5824</td>
<td>67.1</td>
<td></td>
</tr>
<tr>
<td>Coating Wet vs. Dry</td>
<td>0.0063</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Serum x Coating</td>
<td>0.0661</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Trial 1, 2, 3, 4</td>
<td>0.1165</td>
<td>2.18</td>
<td></td>
</tr>
<tr>
<td>Exper. Error</td>
<td>0.0534</td>
<td>5.09</td>
<td>±0.2311</td>
</tr>
<tr>
<td>Subsamp. Error</td>
<td>0.0105</td>
<td>--</td>
<td>±0.102</td>
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</tbody>
</table>
Table 12. Raw Data for Table 11

<table>
<thead>
<tr>
<th>Trial</th>
<th>Patient A Coating</th>
<th>Patient B Coating</th>
<th>Patient C Coating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry</td>
<td>Wet</td>
<td>Dry</td>
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<tr>
<td>1</td>
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<td>1.4783</td>
<td>0.7336</td>
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<tr>
<td>2</td>
<td>1.3927</td>
<td>1.5043</td>
<td>0.7915</td>
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<tr>
<td>3</td>
<td>1.4902</td>
<td>1.6090</td>
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<td>4</td>
<td>1.6039</td>
<td>1.5249</td>
<td>1.0703</td>
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REFERENCES


