DETECTION OF *CANDIDA*

FIELD OF THE INVENTION

The present invention relates to a method and a means of diagnosing *Candida* infection. In particular the present invention relates to a method of diagnosing *Candida* infection which is both sensitive and rapid.

BACKGROUND OF THE INVENTION

Candida is the most commonly identified causative agent of oral or vaginal thrush. However, over the last few decades *Candida* has emerged as a significant cause of life-threatening infections in hospital patients. Ironically the increasing incidence of these "invasive" or "systemic" *Candida* infections has been advances in modern medicine. Patients that are now surviving major injuries, surgery, cancers and organ transplants are vulnerable to life-threatening *Candida* infections. In the United States, *Candida* is now the fourth most common cause of blood infections in hospitals.

The major problem with systemic *Candida* infections is that there are few definitive clinical signs or symptoms. Treatment is largely based on suspicion rather than a definitive diagnosis. Even with the availability of anti-fungal drugs such as fluconazole a high mortality rate (30 to 70%) is associated with systemic *Candida* infections. The high rate of mortality is largely due to the rapid onset of infection and a rapidly fatal outcome. Without an accurate diagnosis the infection often goes unnoticed until it is too late to effectively treat. This has led to a comment by clinicians that *Candida* infections are usually diagnosed at autopsy. Accordingly, there is a need for a rapid diagnostic assay that is capable of early diagnosis of *Candida* infection so that appropriate treatment may be instituted thereby reducing the mortality rate.

The main difficulty in the diagnosis of *Candida*

infections is that being a commensal, mere isolation of *Candida* from body surfaces, or orifices, is not diagnostic of an infection. Culture of *Candida* from blood or deep tissue is still the main method of diagnosis of systemic *Candida* infections. However, it can take several days for a culture to become positive, by then it may be too late to effectively treat the infection. Also, false positives may occur due to contamination from superficial body sites. Of more importance, is the observation that in up to fifty percent of autopsy proven cases of systemic candidiasis, blood cultures were negative and therefore of no diagnostic value.

Nuclear magnetic resonance (NMR) and radioisotope scanning have been used to detect *Candida* infections in tissues and organs. However, those methods are not useful for early diagnosis.

Recently analysis of the *Candida* metabolite arabinitol was proposed as a diagnostic tool. However, as arabinitol is produced by the human body, further clinical studies have cast doubt on its value.

The polymerase chain reaction (PCR) has also been used in the diagnosis of invasive *Candida* infections. However, PCR has not established itself as a useful diagnostic method for *Candida* for the same reasons as outlined above ie *Candida* is a ubiquitously present microorganism and false positives, due to superficial contamination, are prevalent.

Immunoassays are the established procedures for the diagnosis of many types of infectious diseases. Immunoassays have the advantage that they are rapid and have a standardised assay format. Immunoassays can be designed to either detect *Candida* antigens, or host antibodies reactive against *Candida* antigens. Several immunoassays are commercially available for the detection of *Candida* antigens in sera or other body fluids. However, these assays lack either sensitivity or specificity or both.

Immunoassays have been developed based on the detection of immunodominant *Candida* antigens. *Candida* mannan is a highly immunogenic cell wall antigen. However, as *Candida* is a commensal, most individuals have antibody to *Candida* mannan, so its usefulness in the diagnosis of systemic infection is limited. The applicant has now surprisingly found that a more discriminatory assay for *Candida* than previously used is the detection of cytoplasmic antigen. The advantage of this diagnostic assay is that antibody to this cytoplasmic antigen is only produced in response to an actual infection. The applicant has further demonstrated that the use of a combination of cytoplasmic antigen with other antigens is very predicative of *Candida* infection.

Accordingly, the present invention overcomes or at least alleviates the problems normally associated with diagnosing *Candida* infection.

SUMMARY OF THE INVENTION

In its most general aspect, the invention disclosed herein provides a simple and rapid method for diagnosis of *Candida* infection. The method of diagnosis of *Candida* infection may be used to screen large numbers of samples for possible infection.

Accordingly, in one aspect, the invention provides a method of diagnosing *Candida* infection, comprising the steps of:

a). obtaining a biological sample from a subject at risk of, or suspected to be suffering from, *Candida* infection, and

b). measuring the levels of antibody to *Candida* cytoplasmic antigen present in the biological sample.

Antibody levels may be measured using known techniques of immunology including enzyme-linked immunoassay (ELISA or EIA), biligand binding (sandwich technique), fluorometric assay, chemiluminescent assay, immunochromatography, radialimmunodiffusion or

radioimmunoassay (RIA). ELISA, immunochromatography or chemiluminescent assay methods are particularly preferred, since these are quick, sensitive, and specific, and are readily automated for large-scale use. These methods also provide quantitative determinations.

The diagnostic method utilises antigens expressed by *Candida*, especially cytoplasmic antigen. The antigens isolated from *Candida* as disclosed herein may, in certain embodiments of the diagnostic method of the present invention, be immobilised on an inert surface, embedded in a gel, or may be conjugated to a molecule which imparts colour, fluorescence or radioactivity to the antigen.

In a second aspect, the invention provides a method for assessing the prognosis of *Candida* infection, comprising the steps of measuring the levels of antibody to *Candida* cytoplasmic antigen in a biological sample.

Persons skilled in the art will appreciate that the techniques disclosed herein may be used on any type of biological sample. Preferable the biological sample is selected from the group consisting of bone marrow, plasma, spinal fluid, lymph fluid, the external sections of the skin from respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood; both whole blood and sera, blood cells, tumours and organs. Most preferably the biological sample is sera.

Biological samples that may be analysed by the method of the present invention can also be obtained via swabs, shunts or the like. The biological samples may be analysed directly, or may be treated prior to testing by, for example, concentration or pH adjustment.

In a third aspect, the present invention further provides a method of detecting the presence or absence of a *Candida* antibody comprising the steps of:

a). exposing a biological sample, which may include a *Candida* antibody, to an isolated cytoplasmic *Candida* antigen; and

b). detecting the reaction between antibody and

antigen.

In an especially preferred embodiment of the present invention the diagnostic assay further utilises other *Candida* antigens in combination with the cytoplasmic antigen. In particular the cell wall antigen (including mannose) and/or purified immunodominant antigen (enolase) are utilised.

Accordingly, in a forth aspect of the present invention there is provided a method of diagnosing *Candida* infection, comprising the steps of:

a). obtaining a biological sample from a subject at risk of, or suspected to be suffering from, *Candida* infection, and

b). measuring the levels of antibody present in the biological sample to *Candida* cytoplasmic antigen in combination with measuring the levels of antibody to either cell wall antigen or immunodominant antigen (enolase) or both.

The reagents and means of diagnosis of the present invention may also be embodied in a kit for use in a diagnostics laboratory or may be adapted and automated for analysing large numbers of samples at a central receiving centre.

Accordingly, in a fifth aspect the invention provides a kit when used for detecting the presence or absence of a *Candida* antibody in a biological sample, comprising:

a). a biological sample collection device;

b). a cytoplasmic *Candida* antigen; and

c). means for detecting reaction between the antibody and antigen in the sample.

Suitable buffering agents and ionic salts may also be included in the kit.

In a sixth aspect the invention provides a method of preparing a cytoplasmic antigen comprising the step of removing lipoproteins by chloroform extraction.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a coomassie blue stained SDS-PAGE with major protein bands of the *Candida* cytoplasmic antigen fraction observed at 55kDa, 35 to 45kDa region, 30kDa and 20kDa.

Figure 2 shows a single coomassie blue band of 48kDa corresponding to the expected size of the enolase antigen.

Figure 3 shows a coomassie blue stained gel of the clarified cell wall antigen preparation. A broad smear of stain can be seen ranging in size from 90kDa to 200kDa

Figure 4 shows a number of sera screened against the *Candida* cytoplasmic antigen preparation.

Figure 5 shows antibody reactivity to the three Candida antigens - cytoplasmic, cell wall and immunodominant antigens, using negative control sera.

Figure 6 shows antibody reactivity to the three Candida antigens - cytoplasmic, cell wall and immunodominant antigens, using sera from patients with superficial candidiasis.

Figure 7 shows antibody reactivity to the three Candida antigens - cytoplasmic, cell wall and immunodominant antigens, using sera from patients with systemic candidiasis.

Figure 8 shows the error bar of the Applicant antigen test values in the different blood culture patients (95%CI).

Figure 9 shows an error plot of the mean *Candida* antibody values measured by the Applicant antigen test in both the blood culture positive and negative groups of patients (95% confidence interval).

Figure 10 shows an error bar graph of the Applicant antigen test data for invasive candidiasis and healthy controls.

ABBREVIATIONS USED

EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
RIA	Radioimmunoassay
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
β-Me	β-mercaptoethanol
TMB	3,3',5,5'-tetramethyl-benzidine

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The practice of the present invention employs, unless otherwise indicated, conventional molecular biology, cellular biology, and immunoassay techniques within the skill of the art. Such techniques are well known to the skilled worker, and are explained fully in the literature. *See*, e.g., Harlow and Lane, "Antibodies: A Laboratory Manual" (1988); Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "Animal Cell Culture" (R.I. Freshney, ed., 1986); "Immobilised Cells and Enzymes" (IRL Press, 1986); B. Perbal, "A Practical Guide to Molecular Cloning" (1984); Sambrook, et al., "Molecular Cloning: a Laboratory Manual" (1989) and Ausubel, F. et al., 1989-1999, "Current Protocols in Molecular Biology" (Green Publishing, New York).

In describing the present invention, the following terminology is used in accordance with the definitions set out below.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from a individual, including but not limited to bone marrow, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood; both whole blood and anti-coagulated whole blood, blood cells, tumours, organs, and also includes samples of *in vivo* cell culture

constituents, including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively *Candida* infected cells, recombinant cells, and cell components.

"Human tissue" is an aggregate of human cells which may constitute a solid mass. This term also encompasses a suspension of human cells, such as blood cells, or a human cell line.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country.

Persons skilled in the art will appreciate that any number of different immunoassays may be used in the present invention. For example, the *Candida* antigens disclosed herein may be used in antibody capture assays, antigen capture assays, wherein the antigen/antibody complex forms a "special" class of antigen or two-antibody sandwich assays.

TECHNIQUES USED FOR ANTIGEN PREPARATION

The term "*Candida* antigen" as used here means any one of the three separate types of *Candida* antigen utilised in the present invention, namely, cell wall antigen (including mannose), total cytoplasmic antigen (mannose depleted) or purified immunodominant antigen (enolase). Use of the term "*Candida* antigens" means that all three antigens were involved or could be utilised. A number of techniques may be used to prepare the *Candida* antigens including biochemical extraction, column chromatography, Gel fractionation, gene cloning,

differential precipitation, filtration, dialysis or centrifugation; however, the preferred techniques are those disclosed herein. Briefly, these techniques involve either mechanical, chemical or enzymatic lysis of *Candida* cells, followed by separation of insoluble cell walls from soluble cytoplasmic fraction by centrifugation, filtration and dialysis. Chemical treatment of cell wall fraction to release cell wall antigens followed by centrifugation and dialysis. Filtration and organic extraction of soluble cytoplasmic cell extract. Separation of mannoproteins by ConA affinity chromatography. Purification of the immunodominant enolase antigen from the soluble cytoplasmic extract by anion and cation affinity chromatography. It will be appreciated by those skilled in the art that other techniques, or modifications or variations of the above techniques, may be adopted without adversely affecting the spirit of the present invention.

TECHNIQUES USED FOR ANTIBODY PREPARATION AND LABELLING

Antiserum to the *Candida* antigens disclosed herein may be produced in a host animal such as rabbit or sheep. The serum fraction containing the antibody may be isolated by standard techniques. This antiserum may be employed in several of the embodiments of the invention hereinafter set forth, or a more sensitive and specific antibody might be obtained by further purification of the serum by electrophoresis, high-speed centrifugation or the like. Ultimately, large quantities of highly specific monoclonal antibody may be produced by means of the hybrid-myeloma techniques by methods known to those skilled in the art.

Certain embodiments of the present invention employ antibody to the *Candida* antigens immobilised on cellulose, agarose, sephadex or glass beads or other similar inert surfaces such as metal, plastic or ceramic which do not interfere with subsequent reaction. Adsorption, Br-CN activation or other techniques known in

the art may be employed to immobilise the antibody.

Other embodiments of the present invention employ the antibody to the *Candida* antigens conjugated to a chromophoric (highly coloured) molecule, an enzochromic (an enzyme which produces colour upon addition of reagents) molecule, fluorochromic (fluorescent) molecule or a luminogenic (luminescent) molecule.

The conjugate of antibody with enzyme is made using techniques known in the prior art. (For references, see Avrameas, S. and Uriel, J., in Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences, vol. 262, p. 2543, (1966); Nakane, P.K. and Pierce, G.B., in Journal of Histochemistry and Cytochemistry, vol. 14, p. 929, (1966); Nakane, P.K., in Methods in Enzymology, vol. 37, p. 133, (1975)).

Chromophoric molecules that may be used are 2,3-dinitrobenzene (DNB) salts, dinitrophenol (DNP) and methyl and butyl orange. Other suitable chromophoric agents are well known in the art. Enzochromic molecules that may be conjugated with the antibody are enzymes that give colour with appropriate reagents. Examples are alkaline phosphatase (ALP) which develops colour with nitrophenyl phosphate (NPP), glucose oxidase with glucose, and D-galactopyranoside. These and other examples are well known in the art. Examples of fluorogenic agents are 2,4-dinitrofluorobenzene and "pipsyl" derivatives. Luminogenic molecules may be conjugated to antibodies by the method of Branchini, et al. (Biochem. Biophys. Res. Commun. 97, 334 [1980]). The term "chromophoric" hereinafter is intended to include "enzochromic", "fluorochromic" and "luminogenic" molecules as well.

Certain embodiments of the invention also utilise *Candida* antibody tagged with a radioactive element. I^{125} conjugated by means of the chloramine-T procedure is a common example, but other methods known in the art may also be employed.

TECHNIQUES USED FOR ANTIGEN IMMOBILISATION AND LABELLING

Antigen molecules may be immobilised on a solid carrier by a variety of methods known in the art, including covalent coupling, direct adsorption, physical entrapment and attachment to a protein-coated surface. For references describing the methodology, see Silman, I. H. and Katchalski, E. in Annual Review of Biochemistry, Vol. 35, p. 873 (1966); Melrose, G. J. H., in Review of Pure and Applied Chemistry, Vol. 21, p. 83, (1971); and Cuatrecasas, P. and Anfinsen, C. B., in Methods in Enzymology, Vol. 22, (1971).

Lai et al. (German OS No. 2,539,657, U.S. Pat. No. 4,066,512) discloses a method of attachment to a protein-coated surface. In this method, the internal and external surfaces of a microporous membrane are first coated with a water-insoluble protein such as zein, collagen, fibrinogen, keratin, glutelin, polyisoleucine, polytryptophan, polyphenylalanine, polytyrosine, or copolymers of leucine with p-amino phenylalanine. Such a coating renders the membrane capable of immobilising a wide variety of biologically active proteins including enzymes, antigens, and antibodies. A microporous structure is defined as one having more than 50% of its total volume in the form of pores ranging in size from 25 nanometres to 25 micrometers, preferably from 25 nanometres to 14 micrometers. A pore size range from 25 nanometres to 5 micrometers is employed in most applications herein. Uncoated microporous membranes have as much as 70 to 75% of their volume as pore space. The pores permit liquid flow through the membrane. After being coated by zein, for example, the pore space is reduced 5 to 10% with the result that the structure retains its essential properties of having a high proportion of its volume as pore space and permitting liquid flow through the pores. The structure has a large surface area in contact with any solution contained within the pores.

Such a coated membrane, having immobilised antigen or antibody, provides a compact, easy to manipulate carrier for the immobilised antigen or antibody. Its integral structure permits removal of bound from unbound components by simple mechanical means.

Non-specific binding may be minimised by interposing a second stage immobilisation step, in which an immunochemically neutral protein is immobilised to the filter. Immobilisation therefore occurs in two stages according to a preferred embodiment of the invention: a first stage in which the desired immunochemical component is immobilised, and a second stage, following the completion of the first, in which an immunochemically neutral protein such as fetal calf serum or bovine gamma globulin is next immobilised. The term immunochemically neutral is defined in terms of the specific components of the assay. Any protein, which does not combine immunochemically with a component of the assay or with one of the reagents, is considered immunochemically neutral, even though such protein might be immunochemically reactive in another system.

Where the substance to be detected is an antibody, the immunochemically reactive moiety of the conjugate must be an antibody capable of binding immunochemically with the antibody to be tested. Such antibodies may be obtained by immunising an animal with the antibody or immunoglobulin fraction of serum from the animal in which the antibody to be tested originated. For example, where the antibody to be tested is a human antibody, a goat antibody against human antibody is obtained from the serum of a goat immunised against human immunoglobulin (antibody). The enzyme moiety may be any enzyme capable of catalysing a reaction which can be detected by any method known to those skilled in the art, and which retains its activity after conjugation with antibody. Horseradish peroxidase is preferred because of its convenience and suitability to a wide range of

applications. It is well known that the enzyme catalyses the oxidations of a variety of organic compounds in the presence of hydrogen peroxide. Many such organic substrates are chromogenic, ie. undergo a colour change upon oxidation.

It has been found in the present invention that the purity of the enzyme preparation used in the formation of conjugate has an effect on the degree of non-specific binding. The greater the purity of the enzyme preparation, the less the non-specific binding. In part, the reduction is made possible because, the total amount of conjugate protein required is reduced as the specific activity of the enzyme is increased. The opportunity for non-specific binding is therefore reduced as well. In the preferred embodiment, the use of a highly purified peroxidase preparation has been found to significantly reduce the amount of colour reaction observed in control samples as compared with known positives.

TECHNIQUES USED FOR *CANDIDA* ANTIBODY DETECTION

Antibody Capture Technique

A *Candida* antigen prepared by the techniques disclosed herein is immobilised, preferably on an inert surface such as PVC, paper or a similar bibulous mat. The immobilised *Candida* antigen is then put into contact with a sample suspected of containing *Candida* antibody. In the case of aqueous samples such as blood or urine, the solution is buffered and ionic salts may be present at optimum concentration for *Candida* antibody-*Candida* antigen interaction. TRIS or borate buffered phosphate at pH 7.5 to 9.0 and ionic strength about 0.010 to 0.5, for example, are suitable buffering agents and ionic salts. The inert surface with *Candida* antigen or *Candida* antigen-*Candida* antibody complex thereon is next put into contact with antibody to *Candida* antigen conjugated to a chromophormic molecule. Preferably the *Candida* antigen is in solution

buffered at pH from about 7.5 to 9.0 and ionic concentration equivalent to about 0.01M to about 0.1M NaCl. After careful rinsing under water or with suitable surfactants such as Tween 20 to remove excess coloured antibody, the inert surface is inspected for colour, fluorescence or luminescence directly or after addition of colour-developing agents. Colour on the inert surface indicates interaction between immobilised *Candida* antigen-*Candida* antibody complex in solution. A control may be run for colour comparison.

This technique may be adapted to clinical use by employing *Candida* antigens tagged with radioactive elements and observing either depletion of activity in solution or uptake on solid support of radioactivity. This embodiment is highly sensitive and rapid and suitable for large numbers of samples.

Enzyme-Linked Immunoassay-ELISA

A solution comprising *Candida* antibody conjugated to enzyme which forms colour with developing reagents and buffer and ionic salts suitable for reaction between *Candida* antigen and the *Candida* antibody is put into contact and allowed to react with *Candida* antigen immobilised, preferably, on an inert surface such as PVC, paper strip or glass bead. The amount of enzochromic conjugated *Candida* antibody is sufficient to saturate about 50% of the reactive sites on the immobilized antibody. The inert surface with antibody-*Candida* antigen enzyme complex is put into contact with buffered sample suspected of containing *Candida*, said sample having an unknown amount of *Candida* antibody. The colour of the resultant immobilised antibody-*Candida* antigen-enzyme complex on the strip after colour developing reagents are added is observed in comparison to a control strip which has not been treated with sample containing *Candida* antibody. Dilution in colour on inert surface treated with sample means presence of *Candida* antibody in the

unknown sample.

This method may he adapted for clinical use by contacting samples and immobilised enzyme, preferably in tubes which may be centrifuged and watching developing colour spectrophotometrically. This embodiment is very sensitive and rapid.

Radialimmunodiffusion-Precipitin Reaction

One of the *Candida* antigens is suspended in a softened gelatinous medium such as agar or agarose along with buffers and salts to maintain pH between about 6.0 to 9.0 and ionic strength between about 0.01M to 0.5M for optimal antigen-antibody interaction. The suspending medium of U.S. Pat. No. 4,259,207 is a suitable example. The mixture is spread out to harden on a test plate or, preferably, poured into a disc-shaped container such as an Octolony plate. A small amount of sample is placed on the solidified gel, preferably in a centre well and the plate or disc is allowed to stand preferably covered for a period of hours. Diffusion of sample into the surrounding area occurs during this period. If the *Candida* antibody is present, it reacts with the embedded *Candida* antigen and causes an opaque area in a radial pattern about the point of application of sample. A control can be run for comparison. Calibration of an amount of *Candida* antibody in the sample, if desired, can be obtained by controlling temperature, time and size of sample and comparing the resultant size of radial area with one of known concentration.

Radioimmunoassay

A *Candida* antigen of the present invention is immobilised on an inert surface such as glass beads in a separation column. A portion of *Candida* antigen is conjugated to a radioactive element, preferably I^{125} and allowed to react with the immobilised *Candida* antigen in an amount sufficient to, saturate 50% of the binding

sites. The immobilised *Candida* antigen-enzyme complex is put into contact with a sample suspected of containing *Candida* antibody, the sample being buffered between pH 6-9 and containing total ionic salts about 0.05 to 0.5M for optimal reaction conditions for formation of *Candida* antigen-antibody complex. The *Candida* antibody is eluted from the antigen and the eluant is measured for radioactivity. Loss of activity compared to a control indicates *Candida* antibody in the sample.

Haemagglutination

Candida antibody may be assayed through standard haemagglutination techniques with *Candida* antigen to antibody used as sensitising agent.

It is to be understood that methods described hereinabove for assay of *Candida* antibody employing coloured reagents have been presented most specifically for application where neither trained personnel nor sophisticated instruments are available. These methods, however, may be adapted for use in a clinical setting where large numbers of samples are to be assayed by substituting radioactive elements for chromogenic conjugated molecules.

It is also to be understood that the term "colour" is not to be interpreted as being limited to the narrow visible range of the electromagnetic spectrum, but is meant to include wavelengths which may be measured by standard spectrophotographic instruments such as spectrophotometers and absorption and emission colourimeters in both the uv and the ir range.

Although it is contemplated that the methods of the present invention are to be applied to biological fluids themselves, the sensitivity and specificity of the method can be improved by culture of the fluids preferably on medium selective for *Candida* prior to testing.

Sensitivity may also be improved by preliminary treatment of biological samples with lysing agents such as

isotonic solution, sound, or lysozyme to release *Candida* antibody into the extracellular environment. U.S. Pat. No. 4,166,765, for example, discloses suitable lysing procedures for biological samples containing bacteria. Any lysing agent may be employed which does not interfere with subsequent enzyme activity.

ASSAYS EMBODIED IN KIT FORM

The diagnostic method and means of the present invention may be embodied in the form of a kit for use by individuals for self-diagnosis of *Candida* in the privacy of their homes.

The kit comprises a means for sample collection, the *Candida* antigen to *Candida* antibody and a means for detecting reaction between sample and *Candida* antigen.

In embodiments adapted for clinical use, electrophoretic separation techniques such as isoelectric focusing or zone electrophoresis which are based on differences of both size and charge distribution between products and reactants may likewise be used to separate products from reactants. Products separated electrophoretically may be detected by characteristic locations compared to standards or may be identified by colour or immunochemically. Resinous beads of charged surfaces may also be used to separate products and reactants.

The means for detecting reaction in the case of immunoassay in a preferred embodiment of the invention is a gelatinous medium in which the *Candida* antigen to antibody is suspended. The gelatinous medium is in a transparent glass or plastic container and comprises buffer and ionic salts for optimal conditions for formation of the *Candida* antigen-antibody complex. Reaction is noted as a transparent area radiating from the central point at which the sample is applied.

The means for detecting reaction in another preferred embodiment comprising immunoassay is the *Candida*

antigen to *Candida* antibody conjugated to a chromophore in a sealed, sterile packet along with buffer and ionic salts. For assay, the contents of the packet are diluted with water in a marked tube supplied in the kit. Included also in this embodiment is the antigen to *Candida* antibody immobilised on an inert surface. For assay, the inert surface with immobilised *Candida* antigen is put into contact with sample and then with the solution of chromophore-conjugated *Candida* anti-IgA antibody, protein A or protein G. The inert surface is inspected for colour, which indicates *Candida*.

In a particularly preferred embodiment, the kit of the present invention is provided in the form of an immunochromatographic test strip device. There are many patents that cover a number of technologies, formats, reagents and materials that may be of great value in the development and production of immunochromatographic test strip devices. For example, US Patent No. 5,075,078, International Patent Application No. WO95/16207, US Patent No. 5,654,162 and European Patent No. 0810436A1. The assay methods used with the devices disclosed in these patents are essentially the same. A ligand specific for the analyte (normally, but not necessarily an antibody [Ab]) is immobilised to a membrane such as nitrocellulose. The detector reagent, typically an antibody coupled to latex or colloidal metal, is deposited (but remains unbound) into the conjugate pad. When sample (urine, plasma, whole blood, etc.) is added to the sample pad, it rapidly wets through to the conjugate pad and the detector reagent is solubilised. The detector reagent begins to move with the sample flow front up the membrane strip. Analyte that is present in the sample will be bound by the antibody that is coupled to the detector reagent. As the sample passes over the zone to which the capture reagent has been immobilised, the analyte detector reagent complex is trapped. Colour develops in proportion to the amount of analyte present in the sample.

In the present case, while the above principles are the same, rather than detecting analyte *per se*, the immunochromatographic test strip device would detect antibody. In such situations, it would be the antigen(s) disclosed herein which would immobilised onto membranes, sample pads, reagent pads and other porous media rather than antibody. There are a wealth of information regarding the development of such devices including methods of binding antigen/antibodies to nitrocellulose and the like and detecting such bound material. See for example, Towbin *et al*. 1979, Proc. Natl. Acad. Sci. USA 76:4350, the entirety of which is included herein by reference.

Although the invention has been described with reference to presently preferred embodiments, it should he understood that various modifications can be made without departing from the spirit of the invention. Moreover, the following examples are offered by way of illustration only and are not intended to limit the invention in any manner. All patent and literature references cited herein are expressly incorporated.

EXAMPLE 1 PREPARATION OF *CANDIDA* ANTIGEN

The following three types of *Candida* antigen were prepared:

1). Cell wall antigen (including mannose);

2). Total cytoplasmic antigen (mannose depleted); and

3). Purified immunodominant antigen (enolase).

A clinical isolate of the *Candida albicans*, was obtained from a patient with vaginal thrush. The identity of the *Candida* species was confirmed with the use of an API 20C Auxonagram strip (API System S.A., France). The *C. albicans* isolate was designated KEMH5.

200ml YEPD culture medium (1% yeast extract, 2% peptone, 2% D-glucose) was inoculated with the isolate as a starter culture and incubated for 24h at 30°C with

aeration. The starter culture was then used to inoculate a 10L YEPD culture incubated under similar conditions in a 23L Bio-Flo Fermenter IV System (New Brunswick Scientific, Edison, NJ).

The *Candida* culture was harvested from the Bio-Flo fermenter system and separated from culture medium with the use of a Pellicon filtration cassette (Millipore, USA). Concentrated cells were separated from residual medium by centrifugation in 500ml centrifuge flasks for 15min at 1,660 x g and 4°C. The supernatant was discarded and the pelleted cells were resuspended in protein extraction buffer (20mM bis-Tris, pH 6.5). The yeast cells were then centrifuged as described previously, resuspended and pooled for further processing.

Candida cells were ruptured mechanically with the use of a Dynomill® (WAB, Switzerland). Milling was continued until 99% cell disruption was obtained. The soluble *Candida* cell extracts were collected and dispensed into 50ml centrifuge tubes. The extracts were centrifuged for 12h at 8,517 x g and 4°C to precipitate insoluble cell walls. The supernatants containing the soluble cytoplasmic antigen fraction were recovered and passed through a 0.45µm filter membrane.

The filtrates were then extracted with an equal volume of chilled chloroform. Following centrifugation at 4°C for 15min at 1,036 x g the upper aqueous phase was aspirated and transferred to a dialysis tube. The soluble cytoplasmic protein fractions were dialysed in column binding buffer (20mM Tris/HCl, pH 7.4, 0.5M NaCl, 1mM $MnCl_2.4H_20$, 1mM $CaCl_2$) for 12h in preparation for chromatography.

The soluble cytoplasmic antigen fraction was depleted of contaminating soluble cell wall mannoprotein by Con A-Sepharose chromatography. The dialysed cytoplasmic antigen fraction was filtered through a 0.45µm filter. 50ml of the dialysed extract was applied onto a Con A-Sepharose column (2.6 x 12.5 cm) equilibrated in

binding buffer at a flow rate of 4ml/min. The unbound flow-through fraction (non-glycosylated proteins) was collected. Bound mannoproteins were eluted with 0.5M α-methyl mannoside in binding buffer. This step was performed before the next run and to clean the column before storage.

The soluble cytoplasmic antigen fraction was dialysed overnight against 20mM Tris.Cl, pH7.4. An estimate of the quantity of protein in solution was performed using the Bio-Rad® (Bradford) microassay procedure in accordance with the manufacturers instructions. A portion of the cytoplasmic antigen extract was analysed by SDS-PAGE.

As shown in Figure 1 there was a number of major protein bands observed which varyed in size from approx 20kDa up to approx 60kDa in size. The major staining bands being at 55kDa, four bands in the 35 to 45kDa region, 30kDa and 20kDa. This was in stark contrast to the large number of Coomassie blue staining bands in the original crude lysate prior to organic extraction and Con A-Sepharose chromatography.

Purification of the enolase antigen was conducted in the same fashion as the soluble *Candida* cytoplasmic antigen except that it was not subjected to Con A-Sepharose chromatography. Instead, following dialysis and filtering through a 0.20μm syringe filter (cellulose acetate), the filtered extracts were applied to a Pharmacia Biotech XK 50/20 chromatography column packed with Pharmacia Biotech Source 15Q quaternary ammonium anion exchanger (Pharmacia LKB, Uppsala, Sweden). The column was equilibrated prior to chromatography with column binding buffer 'A' (20mM bis-Tris, pH 6.5). Anion exchange chromatography of the crude extracts was controlled and recorded using the Bio-Rad® Econo® system (Bio-Rad Laboratories, USA). Bound protein was eluted from the column with a salt gradient of buffer 'B' (1M NaCl in buffer 'A', pH 6.5). The recovered fractionated

proteins were analysed by an enzyme activity assay.

The active enzyme enolase hydrolyses D(+)2-phosphoglyceric acid (PGA) to phosphoenolpyruvate (PEP). The production of PEP can be monitored by spectrophotometry at 240nm. 20µl of protein solution was combined with 1ml of enolase substrate solution (50mM Tris-HCl pH 7.4, 2.7mM magnesium acetate, 1.0mM EDTA, 1.2mM D(+)2-phosphoglyceric acid) in a quartz cuvette and the change of absorbance recorded at 1min intervals. The specific activity was defined as the conversion of 1µmol of PGA to PEP per min per mg protein. An estimate of the quantity of protein in solution was performed using the Bio-Rad® (Bradford) microassay procedure.

Eluate fractions containing enolase activity were selected and dialysed for 12h at 25°C in hpH_2O. The dialysed fractions were recovered and filtered through a 0.20µm syringe filter. The filtrate was concentrated ten-fold by evaporation under vacuum for 5h. The concentrated samples were dialysed with binding buffer 'A' (10mM sodium acetate, pH 4.7) immediately prior to application to a Pharmacia Biotech Mono S HR10/10 chromatography column packed with methyl sulphonate cation exchanger (Pharmacia LKB, Uppsala, Sweden). Cation exchange chromatography was performed using the Bio-Rad® Biologic system. Bound protein fractions were eluted from the column with a salt gradient of buffer 'B' (1M NaCl in buffer 'A', pH 4.7). Fractions containing enolase activity were identified by the enzyme activity assay described above.

Figure 2 shows a single Coomassie blue band of 48kDa corresponding to the expected size of the enolase antigen. The identification of the 48kDa antigen as the glycolytic enzyme enolase was confirmed by an enolase activity assay.

Purification of the cell wall antigen was conducted as follows: the precipitated insoluble cell walls were collected following centrifugation as described above. The cell walls were washed with hpH_2O then

collected by centrifugation at 6,000 rpm. This step was repeated three times or until the supernatant was no longer cloudy. This ensured any residual soluble cytoplasmic antigen was removed from the cell wall preparation. The washed cell wall pellet was then resuspend in 10mM Phosphate buffer pH7.4 containing 1% v/v β-Me and incubated for 30min at 37°C in a shaker to solubilise the cell wall antigens. The sample was then centrifuged for 5min at 8,000 rpm and the pellet was then discarded. The supernatant was transferred into a fresh tube and recentrifuged (5min at 8,000 rpm). The supernatant containing the solubilised cell wall antigen was then dialysed in hpH_2O for 48h at 4°C (four changes of water), or until no odour was detected. Following dialysis the sample was centrifuged three times 5min at 8,000 rpm to remove any residual particular matter.

Following clarification the cell wall antigen preparation was analysed by SDS-PAGE. The resulting Coomassie blue stained gel is presented in Figure 3. A broad smear of stain is seen ranging in size from 90 kDa to 200 kDa. The lack of discrete protein bands is typical of mannoproteins, where differences in the number of mannose groups added to the protein base results in a variety of molecular weights.

EXAMPLE 2 ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISAS)

A serum panel was collected from 1998 to 2000 from various patients with *Candida* infections. Negative control (Control) sera (n=20) were obtained from the Red Cross Blood Bank, Perth, Australia and was obtained from healthy males in the 19 to 25 year age group. Sera (n=13) from patients with recurrent vulvo vaginal candidiasis (VVC) were obtained from King Edward Memorial Hospital, Perth, Australia. Sera (n=108) from patients with oral candidiasis were obtained from Clinipath Ltd and the UWA Dental School, Perth, Australia. Sera (n=39) from patients (n=28) with systemic candidiasis were obtained

from Princess Margaret Hospital, Perth, Australia and Prince of Wales Hospital, Sydney, Australia.

In the case of patients with oral and vaginal *Candida* infection, confirmation of infection was made by physical examination and by culture of *Candida* organisms from the relevant body site. In the case of patients with systemic infection, confirmation of infection was through positive blood culture or biopsy. In all cases the immune status of the patient was unknown.

Sera from patients with either superficial or systemic candidiasis were screened by ELISA using trays coated with the *Candida* cytoplasmic antigen. The protein content of each antigen preparation was determined using a commercial assay (BioRad) with BSA as a standard. A series of ELISAs were performed to determine the optimal coating concentration for each antigen (data not shown). The optimal coating concentration being that which gave the greatest discrimination between a positive and a negative control serum. For each antigen the optimum coating concentration was determined to be 2μg/ml.

A 96 well C8 strip microtitre plate (Greiner GmbH, Germany), was coated with either *Candida* cell wall antigen, cytoplasmic antigen, or purified enolase antigen as prepared in Example 1. 50μl of a 2.0μg/ml solution of the antigen was diluted in coating buffer (0.1M $NaHCO_3$, pH 9.3) and added to individual wells. The plates were incubated for 12h at 4°C then equilibrated to ambient temperature. After equilibrating the plates to ambient temperature, coating solution was decanted and the plate tapped dried. Plates were inverted on paper towel to drain. Alternatively excess coating solution was aspirated by the automated plate washer (Dynatech Laboratories, Chantilly VA, USA). It was important not to wash the plate at this stage.

A volume of 300μl of blocking solution (PBS pH 7.3, 2% (w/v) BSA (ICN, Australia), 0.01% (w/v) Tween 20), was applied to each well and incubated at 25°C for 90min.

Blocking solution was decanted and the plate tapped dried. Plates were inverted on paper towel to drain and tapped dried for a second time. At this stage plates were either used immediately, or dried for storage. Plates to be dried were placed inverted in a sealable container such as a plastic food container with a number of silica gel desiccant sachets for 48h. The inclusion of approximately 20 small desiccant sachets was adequate for the drying of 6 coated ELISA micro-well trays. Dried plates were sealed into heat-sealed packets with a single desiccant sachet and labelled. Plates were stored at 4°C until required. Packets containing plates were equilibrated to ambient temperature before opening.

Human test sera diluted 1/100 in blocking solution was dispensed into wells in 50µl aliquot's and incubated at 37°C for 30min. The primary antibody solution was aspirated and wells were washed six times in PBS-Tween 20. The plates were inverted on paper towels and allowed to drain for 10min. The plates were then tapped dried.

A volume of 100µl of a horseradish peroxidase anti-human IgG conjugate diluted 1/10,000 in blocking solution was dispensed to each well. Secondary antibody solution was incubated at 37°C for 30min. The secondary antibody solution was aspirated and wells were washed six times in PBS-Tween 20. Plates were inverted on paper towel to drain for 10min and then tapped dried. Plates were inverted on paper towel for a second time and allowed to drain for 5min. Plates were then tapped dried. Particular care was employed to ensure that all traces of secondary conjugate solution was removed as residual conjugate was established as the major factor responsible for disparity of results (Dynatech Laboratories Inc, USA).

A volume of 100µl of TMB liquid substrate solution was dispensed into each well and developed at 25°C for 10min. The reaction was terminated with the addition of 100µl of 1M phosphoric acid or 1M H_2SO_4. The absorbance values for each well were measured at 450nm, reference

620nm with a MRX automated plate reader.

Each immunoassay was performed in triplicate and the mean value of absorbance was used. The absorbances are shown as a Scatter diagram in Figure 4. Three groups of patients with *Candida* infections were analysed. The first group were patients with systemic candidiasis (Systemics), the second group had oral candidiasis (Oral) and the third group had vulvovaginal candidiasis (VVC). Blood bank sera (Control) from males in the 19 to 25 year age group, who were at low risk of having an undetected or subclinical *Candida* infection were used as a control. The cut-off absorbance (OD_{450} = 0.22) was the mean value of the negative control sera. From these data the cytoplasmic antigen ELISA had a sensitivity of 89% and a specificity of 95%. This is higher than that reported for other *Candida* serological tests (Zoller et al., 1991. J. Clin. Micro. 29:1860-1867).

To further increase the sensitivity of the *Candida* ELISA multiple antigens were used. These were the cell wall, cytoplasmic and native enolase (described above).

The use of multiple antigens increased the sensitivity of the *Candida* ELISA. It also provided greater discrimination between superficial and systemic infection. Six negative control sera (serum obtained from healthy males in the 19 to 25 year age group) were used in ELISAs with microtitre tray wells individually coated with the three *Candida* antigens. For each serum the antibody titre to each of the three antigens was below that of the cut-off line (Figure 5). This line is the cut off value assigned based on a comparison of the average antibody titres of sera from control patients versus those of candidiasis patients. The value plotted on the y-axis of the graph is the ratio of the cut-off absorbance divided into the absorbance of the test serum.

Serum obtained from 6 patients with superficial candidiasis was then reacted in the ELISA. Again the

absorbance value of each serum was divided by the absorbance of the cut-off (Figure 6). The characteristic antibody response of the sera from patients with superficial candidiasis was a high titre against the cell wall antigen preparation (1.5 to 2 times the cut-off value). The antibody reactivity to the complete cytoplasmic antigen preparation was positive in most cases (1 to 1.5 times the cut-off). In contrast the antibody titre to the enolase antigen was below or equal to that of the cut-off. There is a correlation between the antibody titre to the internal *Candida* antigens (cytoplasmic and enolase) and the severity of the superficial infection (data not shown). However, the severity of the infection in the six patients analysed was not known.

Six sera taken from patients with systemic candidiasis (confirmed by positive blood culture) were analysed by ELISA. The results are presented in Figure 7. In the case of the patients with systemic candidiasis the antibody response to the cell wall antigen preparation was positive (1.5 to 2 times the cut-off value). Also, the antibody titres to the internal *Candida* antigens (cytoplasmic and enolase) were also positive (1.5 to 2.5 times cut-off value).

CONCLUSIONS

The *Candida* mannan depleted cytoplasmic antigen preparation disclosed herein can be used to identify patients with *Candida* infections. The sensitivity and specificity using an ELISA with microtitre trays coated with this antigen is greater than that obtained by other *Candida* diagnostic tests. Further, the ELISA assay format disclosed herein is easier to perform, more robust and more rapid than formats used in other available *Candida* diagnostic assays. The ELISA format also has the advantage that it is quantifiable. This enables the patient to be monitored over a period of time and changes in the titre of the antibody response to the *Candida*

antigens recorded. The ability of the test to monitor overtime the antibody titre to *Candida* antigens has a prognostic value in terns of measuring the patient's response to antifungal drugs and in the overall survival prospects of the patient. Another advantage of the cytoplasmic antigen preparation is that the method developed to produce the antigen is simpler and more rapid than other available procedures (eg. compare with that of Zoller et al., 1991, *supra*).

EXAMPLE 3 CLINICAL EVALUATION IN FRANCE

Clinical evaluation of the triple antigen test kit as described in Examples 1 and 2 was undertaken in the Department of Parasitology and Medical Mycology at the University of Grenoble Faculty of Medicine, Grenoble, France using stored sera.

Sera from two groups of patients were analysed: those that were blood culture positive and those that were blood culture negative. When possible, sera were taken before, at the time of and after the first day of positive blood culture to be tested. The blood culture negative group was divided into 3 subgroups: Patients that were colonised with *Candida* and were serology positive, patients that were colonised with *Candida* and were serology negative, and patients that were not colonised with *Candida* and serology negative. The sera were obtained from patients hospitalised between 1998 and 2000.

The triple antigen ELIZA test ("the Applicant antigen test") was performed according to Example 2. The cut-off calibrator sera was obtained by pooling sera taken from males in 19 to 25 age group who had no history of *Candida* infections.

Table 1 shows that the Applicant antigen test was positive in 15 out of 19 patients who had a positive blood culture.

TABLE 1

APPLICANT TRIPLE ANTIGEN TEST AS USED IN THE FRENCH STUDY

Patient Group	Patient ID	Serum ID	Candida species	Date of serum relative to first +ve culture	Applicant Triple Ag Abs	Applicant Triple Ag Abs/ Cut off (0.46) ratio	Applicant Triple Ag Score (0.46 cut-off)	Serology-immunofluorescence IFI	IEP Pasteur	IEP FSK	Ag emie
Candidemia	AMI	C1	C.g	-4	1.597	3.5	+++	+++			
	AMI	C2		+3	1.519	3.3	+++	++	+++	+++	
	BRIG	C3	C.a	-2	0.385	0.8	-	-			
	BRIG	C4		+10	0.325	0.7	-	-			
	COE	C5	C.t	-13	0.406	0.9	-	-			
	COE	C6		+1	0.734	1.6	+	+++			
	COE	C7		+29	0.632	1.4	+	-			
	COH	C8	C.g	-14	0.597	1.3	+	-			
	COH	C9		+9	0.661	1.4	+	-			
	COH	C10		+65	0.391	0.9	-	-			
	COM	C11	C.g	+1	1.806	3.9	+++	+	+++	++	
	COM	C12		+19	1.862	4.0	+++	++	++	+++	
	CON	C13	C.g	-27	0.732	1.6	+	-			+
	CON	C14		+1	0.5	1.1	(+)	-			++
	CON	C15		+8	0.367	0.8	-	-			++
	DA SI	C17	C.a	+2	1.805	3.9	+++	+	++++	++	
	DA SI	C18		+70	1.277	2.8	++	++	++	(+)	
	FER	C19	C.a	-35	0.693	1.5	+	-			
	FER	C20		+2	0.368	0.8	-	-			-
	FER	C21		+16	0.229	0.5	-	-			
	FON	C22	C.a	-46	0.51	1.1	(+)	-			
	FON	C23		+3	1.899	4.1	+++	+++			
	FON	C24		+27	1.854	4.0	+++	+++			
	HAM	C25	C.a	+1	1.083	2.4	++	+	+	+	
	HAM	C26		+31	1.168	2.5	++	+	++	(+)	
	HEN	C27	C.t	+2	0.324	0.7	-	-			
	HEN	C28		+7	0.646	1.4	+	-			
	HEN	C29		+40	0.432	0.9	-	-			
	KHA	C30	C.a	-13	0.332	0.7	-	-			
	KHA	C31		+2	1.553	3.4	+++	+			
	KHA	C32		+27	1.393	3.0	+++	+			
	LON	C33	C.a	-2	0.341	0.7	-	-			
	LON	C34		+6	0.447	1.0	(+)	-			
	LON	C35		+61	0.35	0.8	-	-			
	MAN	C36	C.a	-28	0.505	1.1	(+)	-			
	MAN	C37		+5	0.288	0.6	-	-			
	MAN	C38		+72	0.199	0.4	-	-			
	NI	C39	C.t & C.k	+3	0.223	0.5	-	-			
	NI	C40		+9	0.368	0.8	-	++	+	+	

Patient Group	Patient ID	Serum ID	Candida species	Date of serum relative to first +ve culture	Applicant Triple Ag Abs	Applicant Triple Ag Abs/ Cut off (0.46) ratio	Applicant Triple Ag Score (0.46 cut-off)	Serology-immunofluorescence IFI	IEP Pasteur	IEP FSK	Ag-emie
	PAS	C41	C.a	+5	0.865	1.9	+	+	++	+	
	PASe	C42		+32	1.279	2.8	++	+	++	++	+
	PIL	C43	C.p	-2	0.495	1.1	(+)	-			
	PIL	C44		+51	0.831	1.8	+	+			
	RAM	C45	C.t	+5	1.414	3.1	+++	++			
	RAM	C46		+23	1.114	2.4	++	+			
	NOI	C47	?	0	0.611	1.3	+	+++			
	ABE	D21	No info.	No info.	0.748	1.6	+	+			
Hospital	FRE	D22	No info.	No info.	0.454	1.0	-	-			
patients	BEN	D23	C.a	urine	0.331	0.7	-	-			
that are	BER	D24	C.t	mouth/fae	0.463	1.0	-	-			
colonised	BOM	D25	C.a	broncal/fae	1.046	2.3	++	++			
but have	CAP	D26	No info.	No info.	0.658	1.4	+	-			
negative	CAR	D27	C.a	trachea	0.933	2.0	++	+			
Candida	CHE	D28	C.t	urine	1.376	3.0	+++	-			
serology	FER	D29	C.a	urine/faeces	0.363	0.8	-	-			
	GIN	D30	C.g	urine/faeces	0.663	1.4	+	-			
	PER	D31	C.a	thorax drain	0.378	0.8	-	-			
	BEN	D32	C. spp	urine	0.469	1.0	-	-			
Hospital	BON	D33	-		0.44	1.0	-	-			
patients	CIA	D34	C.a	urine	0.92	2.0	++	++			
that are	DAVID CAR	D35	-		0.651	1.4	+	-			
non-colonised	PEL	D36	-		0.752	1.6	+	-			
and have	DI M	D37	-		0.489	1.1	(+)	-			
negative	FEU	D38	-		0.633	1.4	+	-			
Candida	FOG	D39	C.a	thorax drain	1.095	2.4	++	+			
serology	MOR	D40			0.38	0.8	-	-			
	GO	D41			0.677	1.5	+	-			

Patient Group	Patient ID	Serum ID	Candida species	Date of serum relative to first +ve culture	Applicant Triple Ag Abs	Applicant Triple Ag Abs/ Cut off (0.46) ratio	Applicant Triple Ag Score (0.46 cut-off)	Serology - immunofluorescence IFI	IEP Pasteur	IEP FSK	Ag-emie
Hospital patients that are colonised and have positive Candida serology	ALL	D42	C. spp	urine	1.177	2.6	++	+			
	BAR	D43	C.g, C.a, C.t	septic shock	1.375	3.0	+++	+++			
	BOE	D44	C.g	urine/mouth	1.096	2.4	++	++			
	BUI	D45	No info.	No info.	1.125	2.4	++	++			
	COL	D46	No info.	No info.	1.062	2.3	++	+++			
	DAG	D47	No info.	No info.	0.705	1.5	+	-			
	BE	D48	C.a & C. t	mouth/trachea	1.123	2.4	++	+++			
	GEN	D49	No info.	No info.	1.426	3.1	+++	++++			
	GEN	D50	No info.	No info.	1.489	3.2	+++	++++			
	LEC	D51	No info.	No info.	1.668	3.6	+++	+++++			
	LECr	D52	No info.	No info.	1.62	3.5	+++	+++++			

Legend:

Candida Culture Sp.	Applicant Ab	IFI	IEP Pasteur	IEP FSK	Ag-emie
C.a = C. albicans	<10 = -	<20 = -	1 arc = +	1 arc = +	1/2 dil = + 1/4 dil =
C.g = C. glabrata	10-20 = +	20 = +	2 arc = ++	2 arc = ++	++
C.k = C. kefir	20-30 = ++	40 = ++	3 arc = +++	3 arc = +++	
C.p = C. parapsilosis	30-40 = +++	80 = +++	4 arc = ++++	4 arc = ++++	
C.t = C. tropicalis		160 = ++++			
		320 = +++++			

Of the 12 patients who had sera taken before or on the day of the first positive blood culture, 8 gave a positive (or low positive) result. When compared with other serology tests used by the French group 12 out of 19 patients were positive by the immunofluorescence (IFI) serology test. All but one of these was positive using the Applicant antigen test. One patient was also positive by the Applicant antigen test, but negative by IFI. All of the 5 patients that tested positive by IEP Pasteur, IEP FSK or Ag-emie serology tests were also positive by the Applicant antigen test.

It is possible that some of the patients that were negative by both the Applicant and the IFI test may have had a transient candidemia due to central line contaminations.

Six of 11 patients that were know to be colonised, but had negative serology were positive by the Applicant antigen test. Two of the positive patients were also positive by IFI. Of the 10 non-colonised hospital patients with negative serology six were positive by the Applicant antigen test, two of these positive patients were also positive by IFI. All nine patients that were colonised patients with positive serology were positive by the Applicant antigen test. These data compared to 8 out of 9 patients that were positive by IFI. The only IFI negative sample was a low positive by the Applicant antigen test.

The statistical analysis of these data is presented in Figure 8 and Table 2.

TABLE 2

Category	Mean (Units)	95% Confidence Interval
Candidemia Patients	21.79[a,b]	16.25 - 27.33
Colonised + negative Serology	14.55[a,c]	9.67 - 19.42
Non-colonised + negative serology	14.2[b,d]	10.68 - 17.72
Colonised + positive serology	27.27[c,d]	23.12 - 31.43

a. $p = 0.71$
b. $p = 0.58$
c & d. $p < 0.01$

In the candidemia patients with positive blood culture to *Candida*, the mean of their *Candida* antibody levels detected by Applicant antigen test was 21.79 (16.25 - 27.33 95% CI). Using the Independent Samples T-test, the *p* value was 0.71 between the candidemia group and the colonised group with negative serology to *Candida*. The p value was 0.58 between the means of the candidemia group and the non-colonised group that was negative for *Candida* serology.

For the negative blood culture patients, the patients in the groups that were negative for *Candida* serology had generally lower *Candida* antibody levels detected by the Applicant antigen test. The mean antibody levels were 14.55 units in the colonised group (9.67 - 19.42, 95% CI) and 14.2 units in the non-colonised group (10.68-17.72, 95% CI). These levels were significantly lowered ($p<0.01$) from the mean antibody levels in the group of patients that had positive *Candida* colonisation culture results and positive *Candida* serology, mean 27.27 (23.12-31.43, 95% CI). These are clearly seen in the error bars in Figure 8.

Overall, there was a good correlation with the Applicant antigen test and other tests used. There was also a good correlation with the titre of antibody detected by the Applicant antigen test and the level of positiveness of the other tests ie., a patient that had a high positive result with the Applicant antigen test also had a similar result with the other tests used (eg, patients AMI, COM, DA SI, FON, PAS and RAM). Similarly patients that were negative or low positive with the Applicant antigen test were also negative or weak positive by the other tests (eg, patients BRIG, FER, HEN, LON and MAN). It was noted that some of the blood culture negative patients were positive by the Applicant antigen test, which demonstrated the great sensitivity of the Applicant antigen test.

EXAMPLE 4 CLINICAL EVALUATION IN SPAIN

A similar clinical evaluation to that undertaken in Example 3 was conducted by Professors Guillermo Quindós, MD, PhD, María Dolores Moragues, PhD, and José Pontón, PhD, Department of Immunology, Microbiology and Parasitology at the Faculty of Medicine, University of País Vasco, Bilbao, Spain.

The retrospective study sera were obtained from 11 patients (Table 3 - Patients 1.1 to 1.32) with invasive candidiasis as defined by positive blood culture or by histology and positive tissue biopsy. The "blood culture negative" group consisted of sera from 12 patients (Table 4 - Patients 2.2 to 2.53) selected on the basis of the patients have a risk of invasive candidiasis but having negative blood cultures. Between 3 and 5 sera were tested per patient. For patients with microbiologically proven candidiasis the sera were taken before, at the time of and after the positive blood culture. For the blood culture negative group the sera were taken at various times during hospitalisation. As well as sera from hospital patients, sera from three healthy blood donors were also tested (Table 5). Also a group of fresh sera were prospectively tested from 5 patients, two with positive *Candida* blood cultures and 3 without (Table 5).

Table 3

Patients With Positive Blood Culture

Patient	Day extraction	Triple Ag	Platelia Ag	Platelia Ab	Spanish Anti-B	Spanish Anti-GT	Candida species & Outcome
1.01	-21	+	+	+	++++	+	*C. albicans*
1.01	-11	+	+	+	++++	++	
1.01	0	+	+	+	++++	++	
1.01	7	+	++	++	+++	+++	
1.01	13	+++	+	++	+++	+++	
1.11	-1	+++	++	+++	++++	++	*C. glabrata*
1.11	8	+++	++	+++	++++	+++	
1.11	21	+++	+	+	+++	+++	
1.11	29	+++	+	+++	++++	++++	Exitus d45
1.17	-11	+++	++	+++	++++	-	*C. albicans*
1.17	-4	+++	+	+++	+++	+	
1.17	0	+++	+	++	+++	+	
1.17	3	+	+	+	+++	+	
1.17	5	+	+	+	+++	+	Exitus d15
1.18	-6	++	(+)	+	+++	-	*C. parapsilosis*
1.18	2	++	+	+	++	-	
1.18	6	+	+++	+++	+++	-	
1.18	13	+	+++	-	+	+	Exitus d13
1.19	-3	-	-	-	+	-	*C. albicans*
1.19	0	-	+	-	+	-	
1.19	4	-	+	-	+	-	
1.19	7	+	+	-	++	-	Exitus d7
1.22	-11	+	+	+	+	+	*C. albicans*
1.22	-7	+	+	-	+	+	
1.22	0	+	+	+	++	+	
1.22	2	+	+	+	+	+	
1.22	7	+	+	+	+	+	Discharge d7
1.25	-2	-	+	-	++	-	*C. albicans*
1.25	1	+	-	+	+++	-	
1.25	4	+	-	-	++	-	
1.25	14	+	(+)	-	++	-	
1.25	26	+	+	-	++	-	
1.26	-9	-	+	(+)	++	-	*C. parapsilosis*
1.26	-5	+	+	+	+++	-	
1.26	0	+	+	+	+++	-	
1.26	9	+	++	+	+++	-	
1.26	16	+	+	+	++	-	

Patient	Day extraction	Triple Ag	Platelia Ag	Platelia Ab	Spanish Anti-B	Spanish Anti-GT	Candida species & Outcome
1.30	-25	+	(+)	?		+?	*C. albicans*
1.30	-4	+	+	+++		++++	
1.30	0	+	-	+++		++++	
1.30	3	+	-	?		+?	
1.30	56	+	+	?		-?	Exitus d70
1.31	-4	++	-	++		+?	*C. albicans*
1.31	-1	+	+	++		++++	
1.31	6	++	(+)	++		++++	
1.31	13	++	-	++		++++	Exitus d32
1.32	-19	-	-	+		-	*C. parapsilosis*
1.32	-17	-	+	+		-	
1.32	0	-	-	+		-	
1.32	7	-	+	+		-	
1.32	15	-	+	+		-	Discharge d33

Scoring:

Applicant Ab	*Platelia Ag*	*Platelia Ab*	*Spanish B-Ab*	*Spanish GT Ab*
0-10 = -	<0.5 = -	<1=-	*<20*=-	<20=-
10-20 = +	0.5-5=+	1-10=+	*20-80*=+	20-200=+
20-30 = ++	5-10=++	10-20=++	80-600=++	200-600=++
>30 = +++	>10=+++	>20=+++	600-5000=+++	600-1200=+++
			>5000=++++	>1200=++++

Table 4

Patients With Negative Blood Culture

Patient	Day extraction	Applicant Triple Ag	Platelia Ag	Platelia Ab	Spanish Anti-B	Spanish Anti-GT
2.2	1	++	+			-
2.2	5	++	+			-
2.2	6	++	+			(+)
2.4	1	+	(+)			ND
2.4	15	++	+			ND
2.4	17	+	+			ND
2.7	1	+	-	-	+	-
2.7	4	++	+	+	+++	-
2.7	8	+	+	+	+++	-
2.7	11	+	+	+	+++	-
2.7	15	+	+	+	+++	-
2.10	1	+	-		+++	-
2.10	3	++	-		+++	-
2.10	7	++	-		+++	-
2.10	11	++	-		++++	-
2.10	15	++	-		+++	-
2.14	1	++	(+)	+	+++	-
2.14	3	++	-	+	+++	-
2.14	6	++	-	+	+++	-
2.14	9	++	(+)	+	+++	-
2.14	12	++	+	+	+++	-
2.18	1	-	-			-
2.18	4	+	+			-
2.18	8	+	-			-
2.18	12	+	+			-
2.18	22	+	+			+
2.26	1	+	-			-
2.26	9	+	+			+
2.26	16	+	-			+
2.26	23	+	+			+
2.26	30	+	+			+
2.49	1	-	-			-
2.49	11	+	-			-
2.49	15	-	-			-
2.49	18	-	-			(+)
2.49	27	+	-			+

Patient	Day extraction	Applicant Triple Ag	Platelia Ag	Platelia Ab	Spanish Anti-B	Spanish Anti-GT
2.50	1	+	(+)			+
2.50	9	+	+			(+)
2.50	15	+	-			+
2.50	22	+	-			+
2.50	26	+	-			+
2.51	1	-	-			-
2.51	11	-	-			(+)
2.51	18	-	+			-
2.51	22	-	+			-
2.51	29	+	-			-
2.52	1	+	-			(+)
2.52	8	+	-			-
2.52	11	+	-			(+)
2.52	15	+	-			(+)
2.52	18	+	-			(+)
2.53	1	+	-			-
2.53	8	+	-			-
2.53	11	+	+			(+)
2.53	18	+	-			-
2.53	22	+	-			-

Scoring:

Rockeby Ab	*Platelia Ag*	*Platelia Ab*	*Spanish B-Ab*	*Spanish GT Ab*
0-10 = -	<0.5 = -	<1=-	*<20*=-	<20=-
10-20 = +	0.5-5=+	1-10=+	*20-80*=+	20-200=+
20-30 = ++	5-10=++	10-20=++	80-600=++	200-600=++
>30 = +++	>10=+++	>20=+++	600-5000=+++	600-1200=+++
			>5000=++++	>1200=++++

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Table 5

New Patients and Blood Donor Controls

Patient	Day extraction	Blood Culture	Applicant RESULT	Platelia Ag	Platelia Ab	Spanish Anti-B	Spanish Anti-GT
B. Donor 1	N/A	-	-	ND	ND	ND	ND
B. Donor 2	N/A	-	-	ND	ND	ND	ND
B. Donor 3	N/A	-	-	ND	ND	ND	ND
New Patients							
1	N/A	-	-	ND	ND	ND	ND
2	N/A	-	(+)	ND	ND	ND	ND
3	N/A	Asperg	+	ND	ND	ND	ND
4	?	C.g	+	ND	ND	ND	ND
4	?	C.g	+++	ND	ND	ND	ND
5	?	C.a	++	ND	ND	ND	ND
5	?	C.a	+++	ND	ND	ND	ND

Legend:

Culture Species
C.a=*C. albicans*
C.g=*C. glabrata*

Applicant Ab
0-10 = -
10-20 = +
20-30 = ++
>30 = +++

Table 6 summarises the original Spanish data split into the two groups of patients, one blood culture positive and the other blood culture negative. Of the blood culture positive group, the Applicant antigen test identified 8/11 patients as positive before they became blood culture positive. Ultimately 10/11 patients were positive with the Applicant antigen test. Only one patient (1.32) remained negative. This patient was also negative by the Spanish germ tube antibody test and was only transiently positive by the Platelia (BioRad) mannan antigen test. It may be possible that this patient had a transient candidemia.

Table 6

Summary of Spanish Data

	Applicant Triple Ag	Platelia Ag	Platelia Ab	Spanish Anti-B	Spanish Anti-GT
Culture Positive Patients					
Negative result	1	0	1	0	4
Positive before culture	8	11	9	8	6
Positive after culture	2		1	0	1
Total Patients tested	11	11	11	8	11
Culture Negative Patients					
Negative result	0	3	0	0	6
Positive result	12	9	2	3	5
Total Patients tested	12	12	2	3	11
Patient ID					
1.01	pos	pos	pos	pos	pos
1.11	pos	pos	pos	pos	pos
1.17	pos	pos	pos	pos	pos
1.18	pos	pos	pos	pos	(pos)
1.19	(pos)	pos	neg	pos	neg
1.22	pos	pos	pos	pos	pos
1.25	pos	pos	(pos)	pos	neg
1.26	pos	pos	pos	pos	neg
1.30	pos	pos	pos	ND	pos
1.31	pos	pos	pos	ND	pos
1.32	neg	pos	pos	ND	neg
Patient ID					
2.02	pos	pos			neg
2.04	pos	pos			
2.07	pos	pos	pos	pos	neg
2.10	pos	neg		pos	neg
2.14	pos	(pos)	pos	pos	neg
2.18	pos	pos			(pos)
2.26	pos	pos			pos
2.49	pos	neg			(pos)
2.50	pos	(pos)			pos
2.51	(pos)	pos			neg
2.52	pos	neg			neg
2.53	pos	(pos)			neg

The Applicant antigen test identified all 12 of the blood culture negative patient group as being positive for *Candida* antibody. In comparison, the Platelia Mannan antigen test identified 9/12 patients as being positive compared to 5/11 by the Spanish Germ tube antibody test. The main problem with the blood culture negative patient group was that there was no other confirmation of diagnosis.

Overall, there was good correlation with the results of the Applicant antigen test and that of the other serology tests used by the Spanish group. Where a patient was strongly positive by the Applicant antigen test (ie.: patient's 1.11, 1.17, 1.18 and 1.31), they were also strongly positive by the other tests. Also, where sera were negative or low positive by the Applicant antigen test, they are also usually negative or weakly positive by the other tests. For example, patients 1.19, 1.22, 1.25, 1.26, 1.32, 2.18, 2.26, 2.49, 2.50, 2.51, 2.52 and 2.53.

Where fresh sera was analysed (Table 5), there was a perfect correlation with the Applicant antigen test and whether the sera was blood culture positive or negative.

The statistical analysis of these data is presented in Figure 9 and Table 7. From the error plot diagram in Figure 9, it is evident that the group of patients with positive blood culture have a higher *Candida* antibody levels detected by Syscan3 (mean 25.86, 95% CI: 16.28 - 35.44) as compared to the patients with negative blood culture as a group (mean 17.30, 95% CI: 13.42 - 21.19). Comparing the means using the Independent Samples T-Test, the difference between the two groups is statistically significant at $p = 0.087$.

Table 7

Mean and 95% Confidence Interval of Mean of Applicant Antigen Test Scores

Category	Mean (Units)	95% Confidence Interval
Positive Blood Culture	25.86*	16.28 - 35.44
Negative Blood Culture	17.30*	13.42 - 21.19

* p = 0.087

EXAMPLE 5 CLINICAL EVALUATION IN AUSTRALIA

Sera collected from patients with invasive candidiasis was obtained from an Australian hospital (1997 to 1998), the patients had haematological malignancies (n=24). Control sera were collected from males 18 to 25 years of age (n=20) with no history of *Candida* infection. The patient sera were tested with the Applicant antigen test as described in Example 2. Each sera was tested in triplicate and the average reading used. The average absorbance reading for each serum was divided by that of the "cut-off" calibrator serum supplied with the Applicant antigen test. This value was then multiplied by 10 to give a value in arbitrary units.

The results of the Applicant antigen test using a value of 20 units (two times the cut-off calibrator serum value) or above as defining a positive sample is presented in Table 8.

Table 8

Results of the Applicant antigen test using 20 units as a cut-off

	Invasive Candidiasis	Healthy Controls	*Total*
Test Positive	20	0	20
Test Negative	4	20	24
Total	24	20	44

With the Applicant antigen test using 20 units as cut-off, the specificity of the test was 100% and sensitivity was 83.3%. –Positive predictive value was 83.3% and negative predictive value was 100%. When the value of the test considered positive was set at 10 units or 1 times the value of the cut-off sera absorbance, the specificity of the test decreased, but the sensitivity increased (Table 9). The specificity was 90%, sensitivity 87.5%. Positive predictive factor increased to 91.3%, while negative predictive factor decreased to 85.7%.

Table 9

Results of the Applicant antigen test using 10 units as a cut-off

	Invasive Candidiasis	Healthy Controls	*Total*
Test Positive	21	2	23
Test Negative	3	18	21
Total	24	20	44

The results of the Applicant antigen test using sera from patients with invasive candidiasis are presented in Table 10. Only one of the negative results came from a patient who was blood culture positive. Two of the four negative samples were from patients with central line contaminations. Three of the four negative test results came from patients with *Candida parapsilosis* infections, the other being *C. albicans*.

Table 10

Sera from Patients with Invasive Candidiasis Test with Applicant Antigen Test

Patient	Abs (420 nm)	Units	Result	Site of isolation	Candida spp.
A	1.69	61	Positive	blood culture	parapsilosis
B	0.15	5	Negative	blood culture	parapsilosis
C	1.16	42	Positive	peritoneal cavity	guillermondii
D	1.85	67	Positive	blood culture	albicans
E	1.16	42	Positive	blood culture	albicans
F	1.49	54	Positive	peritoneal cavity	glabrata
G	0.56	20	Positive	peritoneal cavity	parapsilosis
I	1.24	45	Positive	cathater	albicans
K	0.98	35	Positive	oesophagus	albicans
L	3.46	124	Positive	peritoneal cavity	albicans
M	0.19	7	Negative	central line	albicans
N	1.44	52	Positive	wound	albicans
P	0.55	20	Positive	sputum	glabrata
Q	1.12	40	Positive	sputum	tropicalis
R	1.02	37	Positive	central line	albicans
S	0.88	32	Positive	blood culture	albicans
T	1.6	58	Positive	blood culture	glabrata
U	0.22	8	Negative	central line	parapsilosis
V	0.55	20	Positive	urine	parapsilosis
W	0.31	11	Negative	peritoneal cavity	parapsilosis
X	0.59	21	Positive	central line	albicans
Y	0.85	31	Positive	blood culture	albicans
Z	1.06	38	Positive	bronch	albicans
ZA	1.05	38	Positive	urine	tropicalis

The Applicant antigen test data for the invasive candidiasis group and healthy controls are presented in the error bar diagram as seen in Figure 10 and Table 11. In Figure 10, the group with invasive candidiasis has a higher mean (31.45 units) as compared to the healthy blood donor group (7.52 units). This difference was statistically significant ($p < 0.01$). The 95% confidence interval range of the means was higher as well in the invasive candidiasis group (23.57 - 39.33 units), as compared to the healthy donor group (6.92 - 8.12 units).

Table 11

Mean and 95% Confidence Interval of the Mean of Patients with Invasive Candidiasis and healthy blood donors

Group	Mean (Units)	95% CI of mean(Units)
Invasive Candidiasis	31.45	23.57 - 39.33
Healthy blood donors	7.52	6.92 - 8.12

In this study the Applicant antigen test was used to test sera from patients with invasive candidiasis, superficial candidiasis (oral or vaginal thrush) and healthy male controls. As a commensal organism, healthy individuals can have a measurable antibody titre to *Candida* antigens. In order to differentiate between normal and infection associated antibody levels a cut-off calibrator serum was supplied. The absorbance of the serum being tested was divided by the cut-off calibrator serum absorbance and multiplied by 10 to give an arbitrary unit value. Using a value of 20 units or above as an indicator of a positive test gave the greatest discrimination between the patient group with invasive candidiasis and the healthy controls (positive predictive value of 83%, negative predictive value 100%). If the value at which a sample was considered positive was lowered to 10 units (ie. the cut-off calibrator value), the positive predictive value increased slightly to 87.5% but the negative predictive value decreased to 90%.

Only one patient with a positive blood culture returned a negative test result with the Applicant antigen test. Two out of the four negative sera were from patients with a central line contamination. This could therefore reflect a transient infection in these patients, which may not provoke an antibody response. It is of interest that 3 of the 4 negative tests were due to *C. parapsilosis* infections. This organism is frequently associated with biofilms, which may shield it from the host immune response.

In conclusion, the Applicant antigen test is a rapid, reliable and easy test to perform. It showed good sensitivity and specificity in the diagnosis of invasive and severe superficial *Candida* infections.

CLAIMS:

1. A method of diagnosing *Candida* infection, comprising the steps of:

a). obtaining a biological sample from a subject at risk of, or suspected to be suffering from, *Candida* infection, and

b). measuring the levels of antibody to *Candida* cytoplasmic antigen present in the biological sample.

2. A method according to claim 1, wherein step b) is by a technique selected from the group consisting of enzyme-linked immunoassay (ELISA or EIA), biligand binding (sandwich technique), fluorometric assay, chemiluminescent assay, immunochromatography, radialimmunodiffusion and radioimmunoassay (RIA).

3. A method according to claim 1, wherein step b) is by ELISA or chemiluminescent assay.

4. A method according to any one of claims 1 to 3, which utilises antigens expressed by *Candida*.

5. A method according to claim 4, wherein the antigen is the cytoplasmic antigen.

6. A method according to claim 4 or claim 5, wherein the antigen is immobilised on an inert surface, embedded in a gel, or conjugated to a molecule which imparts colour, fluorescence or radioactivity to the antigen.

7. A method according to any one of claims 1 to 6, wherein the biological sample is selected from the group consisting of bone marrow, plasma, spinal fluid, lymph fluid, the external sections of the skin from respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood; both whole blood and sera, blood cells, tumours and organs.

8. A method according to claim 7, wherein the biological sample is sera.

9. A method for assessing the prognosis of *Candida* infection, comprising the steps of measuring the levels of

antibody to *Candida* cytoplasmic antigen in a biological sample.

10. A method of detecting the presence or absence of a *Candida* antibody comprising the steps of:

a). exposing a biological sample, which may include a *Candida* antibody, to an isolated cytoplasmic *Candida* antigen; and

b). detecting the reaction between antibody and antigen.

11. A method according to claim 10, further comprising the step of detecting the reaction between further antibody(ies) and other *Candida* antigens.

12. A method according to claim 11, wherein the further antibody(ies) are to the cell wall antigen or purified immunodominant antigen (enolase).

13. A method of diagnosing *Candida* infection, comprising the steps of:

a). obtaining a biological sample from a subject at risk of, or suspected to be suffering from, *Candida* infection, and

b). measuring the levels of antibody present in the biological sample to *Candida* cytoplasmic antigen in combination with measuring the levels of antibody to either cell wall antigen or immunodominant antigen (enolase) or both.

14. A kit when used for detecting the presence or absence of a *Candida* antibody in a biological sample, comprising:

a). a biological sample collection device;

b). a cytoplasmic *Candida* antigen; and

c). means for detecting reaction between the antibody and antigen in the sample.

15. A kit according to claim 14, further comprising buffering agents and ionic salts.

16. A method of preparing a cytoplasmic antigen comprising the step of removing lipoproteins by chloroform extraction.