MATERIALS AND METHODS

3.1 Study Design.

All the isolates included in this study were collected from: 1) Oropharyngeal lesions of the HIV/AIDS patients, 2) diabetic women with vulvovaginal candidiasis. These patients attended out patients department of the All India Institute of Medical Sciences (AIIMS), New Delhi, India. All the specimens for fungal culture were processed in the laboratory for investigation for infection in immunocompromised patient (ICP), department of Medical Microbiology of AIIMS following the standard laboratory techniques (Koneman et al., 1988; WHO protocol, 2001b). The clinical samples were collected as follows:

1. HIV/AIDS patient suffering from suspected oropharyngeal candidiasis 125 HIV/AIDS patients have been studied.
2. Diabetic women with suspected vulvovaginal candidiasis, 200 high vaginal swabs collected during this study.
3. Non-diabetic women with suspected vulvovaginal candidiasis, 150 vaginal swabs from 75 non-diabetic patients. Diabetes mellitus was excluded from the non-diabetic by performing an oral glucose tolerance test as per the criteria of the American Diabetes Association (Gabir et al., 2000). The institutional Ethics Committee approved the study protocol and all the subjects were examined after their informed consent.

3.2 Samples collection.

Samples were collected with sterile cotton tipped swabs sticks. These were moistened in sterile normal saline before collection of samples. Two swabs were taken from each patient, one for direct microscopic examination and other for culture.

3.2.1 Sample Processing.

Smear was prepared with the use of one swab and was stained by Gram stain to see under microscope for the presence of any yeast or any other organism. Other swab was simultaneously subjected to
culture by inoculating on two Sabouraud dextrose Agar (SDA) slants containing 2μg/ml gentamycin and 0.5% cycloheximide and incubated at 37°C for 48 hours.

3.3 Yeast characterization.

The study included:

A) Phenotypic characterizations: -

1) Growth in different media: -
   a) SDA slants (APPENDIX 2).
   b) Cornmeal-Tween 80 agar (APPENDIX 2).
   c) Carbohydrate assimilation test (APPENDIX 3).
   c) Tween 80 Opacity test medium (APPENDIX 3).

2) Morphotyping: -
   a) Staining with Gram’s stain (APPENDIX 1).
   b) Germ tube production test.
   c) Colony morphology.

3) Biotyping: -
   a) Tetrazolium reduction tests (APPENDIX 3).
   b) Urease test (APPENDIX 3).
   c) Carbohydrate fermentation test (APPENDIX 3).

4) Resistotyping.
   a) Antifungal susceptibility testing by NCCLS M27A-2 method.
   b) Spot test.

B) Genotypic characterization: -

1) DNA Fingerprinting of Candida albicans isolates by Southern hybridization.

2) Genotyping of Candida glabrata by arbitrarily primed PCR.

C) Determination of Glyoxylate Cycle enzyme activities.

D) Design a new primer for differential diagnosis of Candida species.
3.3.1 Germ tube test.
Single colony of the yeast isolate was grown on SDA inoculated into test tubes containing 0.5ml of sterile horse serum (Himedia) and was incubated for 2 hours at 37°C. Each batch was checked for germ tube production with a quality control of *C. albicans* (ATCC 2016). Then with a pasture pipette a drop of the sediment was placed on a slide, over-laid with cover slip and examined under a high power microscope.

3.3.2 Growth on Cornmeal (CMA)-Tween 80 agar.
Growth of yeast on CMA reveals distinct morphology regarding blastospores and hyphae development. All the yeast was inoculated by cutting into agar as 4–5 parallel lines approximately 3 mm apart. A flamed sterile cover slip was placed on each surface pattern to produce relatively anaerobic conditions. The plate was incubated at 25oC for 48 hours. The plate was examined under the high power objective for the presence of pseudohypha or true mycelium, blastospores and chlamydomspores. The arrangement of blastospores and mycelia were also observed. The photographs of the *Candida* species grown on CMA have been recorded by using the Zeiss microscope (Axioskop2 MOT model), under magnification power of 40X.

3.3.3 Tetrazolium reduction test (TTC).
The tetrazolium reduction test is a biochemical tests and depends upon the enzyme activity of the yeast grown on this medium. The resultant yeast colony shows different color product. Plates of Tetrazolium reduction medium were streaked by each yeast isolate and incubate at 37°C for 24–48 hours. The plates were then examined for colour and texture of the yeast colonies.
3.3.4 **Urease test.**
In urease test the yeast were lightly inoculated on slants of potato urea agar and incubated at 37°C for 72 hours. The tubes were observed daily for four days to see whether the colour changed to pink or red.

3.3.4 **Carbohydrate fermentation.**
A saline suspension of yeast standard was made, 0.1 ml of this suspension, was not to exceed the turbidity of McFarland 1 fermentation broths. The tubes were incubated at 37°C and observed daily for up to two weeks for acid and gas production.

3.3.5 **Carbohydrate assimilation.**
The carbohydrate assimilation test was done in wells diffusions. A suspension of an isolated colony was made in 2ml of sterile yeast nitrogen base (YNB) that did not exceed the turbidity of McFarland 1 standard. Assimilation of carbohydrate (s) was indicated by growth of yeasts around the wells.

3.3.6 **Tween 80 opacity test and growth on 45°C.**
Tween 80 opacity test was carried out as described by Slifkin (2000) to differentiate the *C. albicans* from *C. dubliniensis* if any among the *C. albicans* strains isolated in this study. *C. dubliniensis* is the yeast associated with oral candidiasis at other clinical sites. This yeast has shared phenotypic similarities with *C. albicans* and thus *C. dubliniensis* may have been misidentified as *C. albicans*. All the strains of *C. albicans* grown on the Tween 80 medium produced a halo response that circumscribed their respective inoculated sites from two to three days post-inoculation. *C. dubliniensis* gave negative response
no halo seen around the inoculation site. Each batch was checked for opacity test with of a quality control of *C. albicans* ATCC 10261.

### 3.3.7 Colonies morphology (Microscopy).

The Direct smear from the clinical samples and the stained smear from culture media examined and photographed using Nikon Labphoto microscope. Zeiss microscope (Stemi 2000-C model) with magnification power of 2.5X was used to study the colony morphology study of *Candida* species. *Candida* species were grown on YPD agar at 37°C for 72 hours. The photographs of the different *Candida* species grown on Tween 80 CMA was done by using the Zeiss microscope (Axioskop2 MOT model) under magnification power of 40X.

### 3.4 Antifungal susceptibility testing by NCCLS M27A-2 method.

Susceptibility of all the isolates to antifungal fluconazole (dissolved in water) (Kind gift from Pfizer, U. K.) was tested following the reference broth micro-dilution method as described in the National Committee for Clinical Laboratory standard (NCCLS) M27-A2 document. The cells grown on Sabouraud's dextrose agar (SDA) were resuspended in 0.9% normal saline so that it attains a turbidity equivalent of 0.5 McFarland. This cell suspension was again diluted serially, first 1:50 in normal saline and then 1:20 in RPMI (Roswell Park Memorial Institute) 1640 media with 0.165 M MOPS (4-Morpholine Propane Sulfonic Acid) buffered to pH 7.0. This diluted cell suspension (100μl) was mixed with serially diluted fluconazole (64μg/ml-0.125μg/ml) in a 96 wells microtitre plate (figure XV), incubated at 35°C for 48 hours. All *C. albicans* isolates resistant to fluconazole were tested for itraconazole (dissolved in DMSO), ketoconazole (dissolved in methanol), and amphotericin B (dissolved in DMSO), by the broth microdilution method as per the NCCLS guidelines (NCCLS-
recommended quality control strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were tested in the same manner and included each time in the experiments. The Minimum inhibitory concentration (MIC) results were recorded by visually using a plate-reading mirror and the cell density was monitored in a micro-plate reader (Bio-Rad model 550, USA) set at 405 nm. MIC was recorded as the lowest concentration of the drug that produced a visible decrease in turbidity compared to that of the drug-free growth control. Figure XVI present the procedure for the MIC test following the NCCLS protocols.
**Materials & Methods**

### RPMI 1640

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**Dilution of Drugs**

+ve Growth control, -ve Sterility control
# Drug at 2X concentration in RPMI 1640.

**Figure XVI: Antifungal susceptibility testing of the yeast.** Scheme shows the procedure for the MIC test following the NCCLS protocols.

The dilution of the drugs, column 11 has no drug (growth control), column 12 is the sterility control of test. Doubling dilutions of drugs are prepared in RPMI 1640. Well 12 of each row is the growth control and contain inoculums without drug. Column 11 has no drug / no inoculums and represents the medium control.
Table XI presents the MIC breakpoints for various antifungal drugs.

Table XI: MIC Breakpoints for various drugs used.

<table>
<thead>
<tr>
<th>DRUG USED</th>
<th>SOLVENT USED</th>
<th>RESISTANT µg/ml</th>
<th>*SDD µg/ml</th>
<th>SUSCEPTIBLE µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>Water</td>
<td>≥ 64</td>
<td>16–32</td>
<td>≤ 8</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>DMSO</td>
<td>≥ 1</td>
<td>0.25–0.5</td>
<td>≤ 0.125</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Methanol</td>
<td>≥ 1</td>
<td>0.25–0.5</td>
<td>≤ 0.125</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>DMSO</td>
<td>≥ 1</td>
<td>0.25–0.5</td>
<td>≤ 0.125</td>
</tr>
</tbody>
</table>

*SDD, susceptible dose dependent.

3.4.1 Spot test:
Ten *C. albicans* strains found to be resistant to fluconazole were rechecked for their resistance to various azoles, i.e. fluconazole, itraconazole and ketoconazole by spot test. Strains were grown overnight on YPD plates at 37°C. Cells were then suspended in normal saline to 0.1 OD (A₆₀₀). 5μl of five fold serial dilutions of each yeast culture were spotted on to YPD plates in the absence (control) of any drug and presence of fluconazole (64µg/ml), itraconazole (1µg/ml) and ketoconazole (0.5µg/ml).

Growth differences were recorded following incubation of the plates for 48 hours at 37°C. Standard strain of *C. albicans* (CAI4) was used as a control for this experiment. Growth was not affected by the presence of the solvents used for the drugs (Mukhopadhyay et al, 2002, 2004).
3.5 CD4+ T-cells count.

In selected number of patients, CD4+ T-cell counts in the blood samples were measured using coulter manual CD4+ T-cell counts kit (Coulter Corporation, Miami, Florida). It was done in 75 patients who had clinical observable oropharyngeal candidiasis and 25 patients who did not have observable oropharyngeal candidiasis lesions. CD4+ T-cell counts were performed by using Coulter Manual CD4+ T-cells Count Kit (Maimai, Fl.) following the manufactures instruction. Figure XVI shows the principle of the Coulter Manual CD4+ T-cells count kit used. The peripheral blood specimens were collected from HIV/AIDS in EDTA liquid and analyzed within 24 hours. EDTA helps to retain cellular integrity well and is used for lymphocyte immunophenotyping. Blood was combined with MY4 Cyto-Spheres Monocyte Blocking Reagent, and then CD4 Cyto-Sphere reagent was added. An aliquot of the mixture was added to a lysing reagent to lyse the erythrocytes, and crystal violet was used to stain the nuclear material of the leukocytes. The lymphocytes coated with CD4-coated latex spheres were counted in a hemacytometer chamber. Complete blood counts were obtained with an automated cell counter. Counting in a hemacytometer identified CD4+ T-cells with beads attached. Cells in all squares on both sides of a 0.1 mm hemacytometer were counted and the number multiplied by the dilution factor of 7.3 to obtain the absolute CD4+ T-cell count.
Materials & Methods

Figure XVII: The principle of the Coulter Manual CD4+ T-cells count kit used in this study.
3.6 Strains and plasmids.

All the yeast strains were isolated in SDA. The bacterial strains carrying the plasmids were maintained in Luria Bertani (LB) broth. All *Candida* species clinical and laboratory isolates were grown in *Candida* strains used in this study were maintained on either YEPD (yeast extract 1%, bactopeptone 2% and glucose 2%) or YNB (yeast nitrogen base w/o amino acids 0.67% and glucose 2%) media, as required. When grown on solid media, 2.5% agar was added to both the media. pRFL37 (carrying CARE-2) fragment was a kind gift from Dr. B.A. Lasker (Centers for Disease Control, Atlanta). All the oligonucleotides used obtained from Sigma Co.

Clinical isolates *C. dubliniensis* was obtained as kind gift from Prof. Yuzuru Mikami, Japan. Table XII shows the list of the standard strains used as controls in this study obtained from American Type Clinical Collection (ATCC).
Table XII: List of the standard strains used as controls in this study obtained from American Type Clinical Collection (ATCC).

<table>
<thead>
<tr>
<th>Candida species</th>
<th>ATCC Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parapsilosis</td>
<td>22019</td>
</tr>
<tr>
<td>C. krusei</td>
<td>6258</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>750</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>90030</td>
</tr>
<tr>
<td>C. dubliniensis</td>
<td>MAY-646</td>
</tr>
<tr>
<td>C. albicans</td>
<td>90028</td>
</tr>
<tr>
<td>C. albicans</td>
<td>10261</td>
</tr>
<tr>
<td>C. kefyr</td>
<td>2512</td>
</tr>
<tr>
<td>C. utilis</td>
<td>34438</td>
</tr>
<tr>
<td>C. pelliculosa</td>
<td>8168</td>
</tr>
</tbody>
</table>

3.7 Extraction of genomic DNA from Candida species.

The Candida species cells were allowed to grow in 15 ml of YPD broth for 12–16 hours were harvested by centrifuge at 3000 rpm for 5 minutes and were resuspended in 1 ml of SOE (1M Sorbitol and 0.1 M EDTA). SOE was then added to the cells to make final volume of 500 μl and 20 μl of 10-mg/ml solution of Zymolyase was added and the tubes incubated at 37°C. The tubes were then turned upside down gently after every 15 minutes. The contents of the tube were then centrifuged at 2000 rpm for 1 minute until the cells were pelleted enough. The supernatant was discarded and the cells were resuspended by vortexing thoroughly for one minute in 500 μl of 50 mM Tris, 20-mM EDTA mix. 50 μl of 10% SDS was added to the above suspension and incubated at 65°C for 30 minutes. 200 μl of cold 5.0M potassium acetate was added and the tubes were chilled on ice for 60 minutes after vortexing to mix.
the contents. After incubation on ice the cell suspension was centrifuged for 5 minutes at 4°C. The supernatant was recovered in fresh tubes after centrifugation and 1 volume of isopropanol was added. The tubes were inverted several times to get a pellet of DNA. Eventually centrifuging briefly the pelleted DNA down. The pellet was semi dried in a speedvac and resuspended in 150 μl of TE. The tubes were kept at 65°C till the DNA dissolved completely. 1.5 μl of RNase A solution (10 mg/ml) was added and the tubes were incubated at 37°C for 30 minutes. The tubes were chilled on ice and then centrifuged for 5 minutes at 4°C. The supernatant was transferred to fresh tubes and the DNA was precipitated with 2.5 volumes of ethanol and 1/10 volume of 3.0 M sodium acetate (pH 5.2). The DNA pellet was washed with 70% ethanol and dried in a speedvac. The DNA pellet was finally resuspended in 100–200 μl of sterile TE or MQ water.

3.7.1 DNA Fingerprinting of the Isolates by Southern hybridization. Chromosomal DNA was isolated from each isolate using conventional technique after lysis of the cells by Zymolyase (Seikagaku, Japan) treatment. Around 2 μg chromosomal DNA from each isolates was digested with restriction enzyme EcoRI to completion. Digested DNA samples were then separated on agarose gel (0.8%) in 1x TBE buffer (89 mM Tris-borate, 1mM EDTA) by applying a voltage gradient of 2 volts/cm for a period of 20 hours, stained with Ethidium bromide (0.5(g/ml), destained, visualized under UV and photographed using gel documentation system (Alpha Innotech, USA). Separated DNA fragments were denatured in situ using alkali and then neutralized with acid. The denatured DNA fragments were then transferred to nylon membrane (Sigma) by capillary action. Transferred DNA fragments were then UV cross linked (Stratagene, USA) to the membrane and prehybridized in 300 mM phosphate buffer containing 7% SDS and 1
mM EDTA at 65°C for 2–4 hours. In the next step immobilized fragmented DNA were hybridized with \(^{32}\text{P}\)dATP (Amersham Pharmacia Biotech, U. K.), labeled C. albicans specific probe CARE-2 (kind gift from Dr. B. A. Lasker, Centers for Diseases Control, Atlanta) (Lasker et al, 1992), at the same temperature for 16 hours. The nylon membrane containing the hybridized DNA fragments were then washed several times with 2x SSC containing 0.1% SDS exposed to X-ray film at \(-80^\circ\text{C}\) for 16–24 hours and developed.

3.7.2 Bacterial miniprep DNA isolation.
3ml of bacterial culture (harboring the desired plasmid) was harvested at 6000 rpm for 5 minutes. Plasmid DNA was isolated using alkaline lysis method (0.2N NaOH and 1.0% SDS). Contaminating proteins and *Escherichia coli* genomic DNA were removed by extraction with phenol: chloroform: isoamyl alcohol (25:24:1) twice. The DNA was precipitated with 2.5 volume of chilled ethanol. Precipitated DNA pellet was subjected to 70% ethanol wash and then dried under vacuum and finally resuspended in 50.0 µl of TE buffer (10.0 mM Tris-Cl pH 8.0, 1.0 mM EDTA).

2.7.3 CARE-2 probe.
CARE-2 (*C. albicans* repetitive element -2) species-specific probe is a middle repetitive element isolated from the genome of pathogenic yeast *C. albicans* and is present a minimum of 10 to 14 copies per haploid genome. CARE-2 is interspersed and shows a high degree of RFLP. The CARE-2 nucleotide sequence is characterized by cluster of six short perfect direct repeats of 6 bp in length. CARE-2 is 1.06-kb sequence represents a unique family of DNA sequence. Figure XVIII present the preparation of CARE-2. It can be used for epidemiological or taxonomic investigations. It is dispersed on all chromosomes, and southern blots hybridization demonstrates different copy numbers on different
chromosome (Lasker et al, 1992). CARE-2 distinguished unrelated isolates and identified the same strain in independent isolates (Soll, 2000).

**Figure XVIII: Preparation of CARE-2 fragment.** CARE-2 probe can be excised from plasmid pRFL37 using KpnI and PstI, which flank the BamH1 site of the vector yielding a 1.06 kb band.

### 2.7.4 Labeling of CARE-2 Probe.

For labeling of CARE-2 Probe About 5–10μg of plasmid DNA (pRFL37) containing the CARE-2 sequence was digested with restriction enzyme KpnI and PstI (figure XVII) (Lasker et al, 1992) and the generated DNA fragments were separated on agarose gel (1%) in 1x TBE buffer. The 1.06 Kb DNA fragment containing the CARE-2 sequence was then cut out of the gel, smashed, suspended in 1x TE buffer and purified out of agarose. The purified DNA fragment was hybridized to random primers
(Takara, Japan) and extended in presence of 200 µM each of dGTP, dCTP, dTTP & 10 µCi [α-32P]dATP (6000 Ci / mmol.), by Klenow fragment at 30°C for 30 minutes, labeled DNA fragment was then ethanol precipitated and washed several times with 70% ethanol to get rid off unincorporated nucleotides. Finally the precipitated DNA was dissolved in hybridization buffer and used in subsequent step.

2.7.5 Computer system to analyze DNA fingerprint patterns.
There are number of software programs available for generating dendrograms from matrices automatically or manually entered. DENDRON developed at the University of Iowa; combine the capabilities of image processing, gel image analysis, computation of similarity coefficients, genesis of dendrograms, and storage of the data for future retrospective analysis.

3.7.6 Dendrogram and Cluster analysis.
Dendrogram was generated by comparing the relatedness of DNA band pattern in the autoradiogram, by computing the similarity coefficient (S_{AB}) of the bands, using the DENDRON software package, version 3.0 (Solltech, Iowa City, Iowa), as described by Pujol et al, (1997).
The autoradiogram images of Southeren blot hybridization patterns were digitized into DENDRONE data file with Scanjet 3500 scanner (Hewlett- Packard Co., Palo Alto). Distortions in the gel were removed and each lane was automatically identified and scanned. After verification of the identified bands, they were edited manually, and matrices were generated on the basis of band position (molecular weights). From this information, band data files containing the genetic makeup of each isolate (i.e., the presence or absence of bands) were generated. Using these band data files, S_{AB} values were then computed and dendrograms based on S_{AB} were generated. The steps of the preparation of dendrogram are described in Figure XIX.
Figure XIX: Image-processing and analysis capabilities of a computer-assisted DNA fingerprinting analysis program DENDRONE. (1) Original digitized image with distortion. (2) The program straightens the gel according and align the lanes. (3) The program automatically identified lanes and bands and uses the pixel density to assign intensity class. (4) The program then correlates bands with universal standard. (5) The program generates a model. (6) The program then processed the gel image and the Dendrogram generated.
3.8 Molecular genotyping of *C. glabrata* by arbitrarily primed PCR.

Amplification reactions were performed in 100 μl of a PCR mixture containing 100 μM (each) dATP, dCTP, dGTP, and dTTP, enzyme buffer, 50 pmol primer and 2.5 U DNA Taq polymerase (NEW ENGLAND, Biolabs). The sequence of the primer used is: 5'GATTCAGACC-3' as it has been characterized by Becker *et al* (2000). The amplification was performed in an automated thermocycler (iCycler, Bio-RAD). The thermal cycler conditions were 30 cycles of denaturation at 94°C for 1 minute (2 minutes for the first cycle), annealing at 35°C and polymerization at 72°C for 2 minutes. Amplified products (25 μl) were resolved by 1 % gel electrophoresis at 100 V for 1.5 hours the gel was stained with ethidium bromide, exposed to UV light to visualize the amplified products and photographed by using the AlphaImager™ IS-3400 Alpha Innotech.

3.9 Determination of glyoxylate cycle enzyme activities.

Measurement of the enzyme activities of glyoxylate cycle were carried out by spectrophotometric methods using a BECKMAN DU-68 recording spectrophotometer by taking the reduction or oxidation of NADP and NAD by coupling the reaction using purified enzymes, and the rate in the change of optical density was measured for 4–5 minutes at 340 nm except for isocitrate lyase which was measured at a wavelength of 324 nm.

3.9.1 Preparation of cells extracts.

*C. albicans* was grown in yeast extract peptone dextrose broth (Yeast extract 1%, Peptone 2%, Dextrose 2%) at 30°C in a shaking incubator for 24 hours. Cells from culture were harvested by centrifugation at 87
3000 rpm for 10 minutes, washed once with 100 mM potassium-phosphate buffer (pH7.5, 4°C) and resuspended in 100 mM potassium-phosphate buffer, pH 7.5, containing in final concentration 2mM-MgCl₂ and 1mM-dithiothreitol. The extracts were prepared immediately after washing by potassium-phosphate buffer; the cells were disrupted with 0.5-mm diameter glass beads at 4°C for 1 minute using fast-prep apparatus (FP 120, Savant). Whole cells and debris were removed by centrifugation at 10,000 rpm for 10 min at 4°C using a refrigerated centrifuge. The clear supernatant, containing 2–4 mg protein ml⁻¹, was used as cell-free extract for all the enzyme estimations.

The level of enzyme activities were estimated in: (a) eight isolates from HIV/AIDS patients (four fluconazole resistant and four fluconazole susceptible), (b) twelve isolates from diabetic patients (six fluconazole resistant and six fluconazole susceptible) (c) eight isolates from burn patients (four fluconazole resistant and four fluconazole susceptible) and (d) four strains of C. albicans were used as control (C. albicans ATCC 10261, three isolates of normal flora isolated from asymptomatic individuals). In order to examine the reproducibility of the results, enzyme estimations including all other procedures were carried out in triplicate for each clinical isolate.

3.9.2 Isocitrate lyase (EC 4.1.3.2).
Isocitrate lyase catalyses the following reaction:

Isocitrate $\xrightarrow{\text{Isocitrate lyase}}$ Succinate + glyoxylate

The isocitrate lyase activity was measured by the method described by Jong-Gubbels et al [1995] based on the spectrophotometric detection of glyoxylate phenylhydrazone. The reaction mixture in a volume of 1ml contained the following in the final concentration potassium-phosphate buffer (pH 7.0) 100 μmole, phenyl hydrazine 4μmole, cysteine 2.5μmole, MgCl₂ 2.5 μmole, and 2 μmole of D, L-isocitrate. The reaction was
Materials & Methods

started with the addition of the cell-free extract containing approximately 400µg protein.

3.9.3 Malate synthase EC 2.3.3.9 (formerly EC 4.1.3.2).
The following reaction is catalyzed by malate synthase.

\[
\text{Malate synthase} \quad \text{Acetyl-CoA} + \text{H}_2\text{O} + \text{glyoxylate} \rightarrow \text{S-malate} + \text{CoA}
\]

Malate synthase activity was measured by the method described by Jong-Gubbels et al, [1995]. The reaction mixture in a volume of 1ml contained the following in the final concentration of Tris HCl buffer (pH 8.0), 100 mM, acetyl-CoA 0.4µmol, MgCl₂ 5 µmole, NAD 0.4 µmole, citrate synthase 2U, malate dehydrogenase 12U, and 0.25 µmole sodium glyoxylate. The reaction was started with the addition of the cell-free extract containing approximately 400µg protein.

3.9.4 Malate dehydrogenase (EC 1.1.1.37).
The following reaction is catalyzed by malate synthase.

\[
\text{(S) Malate} + \text{NAD}^+ \rightarrow \text{Malate dehydrogenase} \rightarrow \text{Oxaloacetate} + \text{NADH} + \text{H}^+
\]

Malate dehydrogenase activity was measured by the method as described earlier by Thakran et al, (2003) by following the decrease in optical density at 340 nm. The reaction mixture in a volume of 1ml contained the following in the final concentration of Tris HCl buffer (pH 8.0) 100 mM, MgCl₂ 5µmole, Oxaloacetate 0.76µmol, and NADH (0.1 ml of 2mg/ml) 2.7mM. The reaction was started with the addition of the cell-free extract containing approximately 100µg protein.
3.9.5 Citrate synthase EC 2.3.3.1 (formerly EC 4.1.3.7).

The following reaction is catalyzed by citrate synthase:

\[
\text{Citrate synthase} \quad \text{acetyl Co-A} + \text{H}_2\text{O} + \text{oxaloacetate} \rightarrow \text{Citrate} + \text{CoA}
\]

Citrate synthase was measured by the method described by Jong-Gubbels et al., [1995]. The reaction mixture in a volume of 1ml contained the following in the final concentration of Tris HCl (pH 7.6), 100 Mm, NAD 8 µmole, acetyl Co-A 10 µmole, malic acid 0.05 µmole, and malate dehydrogenase 1.5U. The reaction was started with the addition of the cell-free extract containing approximately 400µg protein.

3.9.6 Estimation of Protein.

Soluble protein was determined by the Coomassie blue method of Bradford, (1976) using bovine serum albumin (BSA) as standard. This method involves the binding of Coomassie Brilliant Blue G-50 to protein, causing a shift in the absorption maximum at 595 nm, which is monitored. The color reagent is prepared by dissolving 100 mg of Coomassie Brilliant Blue G-50 in 50 ml of 95% ethanol to which 100ml of 85% (v/v) orthophosphoric acid was added, and the volume made up to 1 liter. Standard protein solution (BSA) was prepared containing 10 to 100 µg of protein in a 100µl volume was pipetted into separate test tubes. The volume in the test tube was adjusted to 100µl by the addition of distilled water, 5 ml of coloring reagent was added to the test tubes and the contents were mixed by vortexing. The absorbance at 595 nm was measured after 2 minutes. A standard graph was made using 1 mg/ml BSA solution. The amount of protein in the sample was estimated by extrapolating the value of the absorbance of the sample from the standard graph.
3.10.6 Calculation and definitions of enzyme unit.

The enzyme activities were calculated by taking the molar extinction coefficient of the NADH, NADPH as $6.22 \times 10^6$ cm$^2$ / M. An absorbance change of 6.22 in 1.0 ml reaction mixture would imply reduction/oxidation of 1 µmole of NAD (P)$^+$ /NAD (P) H corresponding to 1 µmole conversion of the substrate into product. The equation for calculation specific activity in units per mg of cells per minute is as follows:

$$\text{Absorbance change/min} \times \text{dilution of extract} \over \text{Volume of extract} \times 6.22 / \text{total reaction volume}$$

Enzyme units/gm/min =

For example, the activity of malate synthase was calculated as following:

Total reaction volume = 1.0 ml
Volume of extract=0.05 ml
Dilution= 10
Change in the absorbance/minute (Δ O. D.) = 0.0122

$$0.0122 \times 10$$

Activity of isocitrate lyase =

$$0.05 \times 6.22 / 1$$

= 0.39 units/mg/minutes.
3.10.7 Statistical analysis.

Chi-squared test was used to compare the incidence and drug susceptibility pattern between diabetic and non-diabetic women. Results of glyoxylate enzyme activity are presented as mean ± SEM of more than four separate experiments. The significant difference between the data pairs was evaluated by analysis of variance (ANOVA) test followed by student-Newman-Kelus multiple comparison tests were employed for statistical comparison. The significance level was considered at $P < 0.05$.

All the restriction and modifying enzymes were from Roche Molecular Biochemical. Random primer labeling kit and PCR amplification kits were procured from Gibco BRL and Roche, respectively. Zymolyase 100T was from Seikagaku Corp., Japan. Coelenterazine was purchased from Molecular Probes Inc. All the media chemicals were from Difco, USA or Himedia, India. All the other chemicals were procured locally and were of molecular biology grade. All the purified enzymes, coenzymes, substrate, standard and buffers were purchased from Sigma chemicals company (St. Louis, M. O., USA).
3.11 Diagnostic Random Amplification Polymorphism DNA (RAPD)

Fifteen random oligonucleotides have been designed in this study and obtained from Sigma ALI-1 to ALI-15 (table XIII). These single primers are composed of 10 bases and containing various percent GC contents (table XIII). These primers then tested for primary screening of 100 unrelated isolates of Candida species. First, preliminary trails with different annealing temperatures (30, 32.5, 35, 37.5 and 40°C), cycle number (20, 25, 30, 35, 40), different units (1.0, 1.5, 2.0, 2.5, 3.0) of DNA Taq polymerase. The primer (ALI-8) generating the species-specific pattern was tested using different isolates of Candida species along with the standard strains of American Typed Clinical Collection (ATCC). To standardize the test the RAPD test as been carried out in different thermal cycler machines.

Different PCR conditions used to optimize the better conditions for better amplicons products. PCR carried out with about 50 ng of DNA; 200 μM (each) dATP, dCTP, dTTP, and dGTP; 50pmol of oligonucleotides; 0.5 U of Taq polymerase (NEW ENGLAND, Biolabs); and ThermoPol reaction buffer (1 X ThermoPol reaction buffer: 10 mM KCl, 10 mM (NH₄)₂ SO₄, 20 mM Tris-HCl, 2 mM MgSO₄ and 0.1 Triton X-100, pH 8.8 at 25°C). The final volume of the reaction was 50 μl.
Table XIII: The random oligonucleotides designed and used in this study.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Name</th>
<th>Sequence (5’– 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ALI-1</td>
<td>CCGATTCGGG</td>
</tr>
<tr>
<td>2</td>
<td>ALI-2</td>
<td>CAACGCTTAGG</td>
</tr>
<tr>
<td>3</td>
<td>ALI-3</td>
<td>TCCCCAGTAC</td>
</tr>
<tr>
<td>4</td>
<td>ALI-4</td>
<td>GTCCGGATGA</td>
</tr>
<tr>
<td>5</td>
<td>ALI-5</td>
<td>TGACGACTTC</td>
</tr>
<tr>
<td>6</td>
<td>ALI-6</td>
<td>GAACTGCCTG</td>
</tr>
<tr>
<td>7</td>
<td>ALI-7</td>
<td>AACGTGCTGA</td>
</tr>
<tr>
<td>8</td>
<td>ALI-8</td>
<td>CCTTGACGCA</td>
</tr>
<tr>
<td>9</td>
<td>ALI-9</td>
<td>CTAGACCGAT</td>
</tr>
<tr>
<td>10</td>
<td>ALI-10</td>
<td>GGTGGTGGTA</td>
</tr>
<tr>
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<td>ALI-11</td>
<td>CCGCGCCCGC</td>
</tr>
<tr>
<td>12</td>
<td>ALI-12</td>
<td>GCTCGGCACC</td>
</tr>
<tr>
<td>13</td>
<td>ALI-13</td>
<td>GCATCAGCTG</td>
</tr>
<tr>
<td>14</td>
<td>ALI-14</td>
<td>GGTTCGCGGCA</td>
</tr>
<tr>
<td>15</td>
<td>ALI-15</td>
<td>CTAAGTCTGG</td>
</tr>
</tbody>
</table>
3.11.1 The DNA thermal cycler was programmed with conditions.

All the experiments were repeated more than five times for each isolates to assess the reproducibility of the technique. Different PCR conditions used to optimize the better conditions for better amplicons products. The well again conditions in which we have found the primer gave best discrimination power among different Candida species. The cycling conditions were 94°C for 2 minutes, then 30 cycles of denaturation at 94°C for 1 minute, then 30 cycles of denaturation at 94°C for 1 minute, polymerization at 72°C for 2 minutes. Amplified products (25 μl) were resolved by agarose 1% gel electrophoresis at 80 V for 1.5 hours. The gel was stained with ethidium bromide then visualized under UV and photographed using gel documentation system (Alpha Innotech, USA).

In all the experiments positive and negative controls were used. After standardization the technique the RAPD technique carried out with number of isolates on coded bases. The experiments were carried out blindly with out knowing the identity of the isolates. Later we received three isolates from hospital, conventional identification of the isolates couldn’t identify following the conventional methods and by this procedure we could reach the diagnosis of the isolates.