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Comparison between culture and non-culture based methods for detection of Nosocomial fungal infections of *Candida spp*.in intensive care unit patients

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ABSTRACT

The main objective of the present study was to compare between culture based method (Hicrome Candida Differential Agar) and non-culture based method (PCR) for detection and identification of Candida spp. (*C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis*) isolated from intensive care unit (ICU) patients of New Kasr El Aini Medical School Educational Hospital, Egypt. The study was carried out during the period August 2008 to September 2009. The results showed that Candida isolates that isolated from Hicrome Candida Differential Agar plates were positive with PCR technique as follow: 100, 95.0, 85.0 and 90.0 for *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis,* respectively. This means that using of Hicrome Candida Differential Agar media for detection and enumeration of Candida spp. was highly sensitive, rapid and economic method.

Key word: Nosocomial fungal infections, *Candida spp*

INTRODUCTION

Candida species are capable of causing superficial mucosal lesions in both the oral and vaginal cavity when the balance between host and fungus shifts in favour of the fungus. Candida spp. is part of the normal flora on the skin and on the mucosal membranes of the oral cavity and gastrointestinal tract. Candida spp. can be recovered from sputum in 20% of health care personnel and 55% of hospitalized patients receiving antibiotics (Baum, 1960; Meersseman *et al*., 2009).

Candida infections are generally endogenous in origin, and prior colonization with the organism is often regarded as one of the major risk factors for candidiasis (Pfaller 1995; Pfaller and Diekema 2007). Colonization rates are higher in individuals whose mucosal immunity is impaired due to old age, diabetes mellitus or smoking (Lockhart *et al*., 1998; Manfredi *et al*., 2002).

Moreover, *C. albicans* is by far the most common cause of mucosal yeast infection, being the sole species recovered from up to 70% of HIV infected individuals and up to 90% of cases of Candida vaginitis (Coleman *et al*., 1993; Sobel, 2007). Other Candida species can be recovered alone or co-isolated with *C. albicans* from sites of mucosal infection (Coleman *et al*., 1995).

The presumptive clinical identification of *C. albicans* is usually made on the basis of its ability to generate germ tubes when incubated at 30 to 37°C for 2–4 h in serum. This germ tube production in *C. albicans* is affected by various environmental conditions. Although the germ tube (GT) test is an economic, easy, and rapid method available for screening for *C. albicans*, up to 5% of *C. albicans* strains are germ tube-negative (Perry and Miller, 1987) and false positive results can occur with certain

non-albicans yeasts, such us *Candida tropicalis* or *Candida parapsilosis* (Freydie´re and Guinet, 1997).

These problems imply that a well-trained laboratory staff in clinical mycology is strongly required. There is an obvious need for rapid and cost-effective differentiation of *C. albicans* from other sometimes drug-resistant Candida species in clinical microbiology (Odds, 1993). Also, reliable detection of mixed cultures might improve therapeutic intervention (Ainscough & Kibbler, 1998). In response to this increased need, several commercial systems are now available. Albicans ID2, Chromalbicans Agar, and CHROM agar Candida contain chromogenic or fluorogenic substrates hydrolyzed by the hexosaminidase of *C. albicans*. This leads to a rapid identification of *C. albicans* on the basis of colony colour. These substrates offer rapid identification directly upon primary culture (Carrillo-Mun˜oz *et al*., 2001; Ca´rdenesa *et al*., 2002).

There are several chromogenic media available for the isolation and presumptive identification of *C. albicans* based on the pigmentation of the developing colonies, which is due to different enzyme activities among *Candida* species (Baumgartner *et al*., 1996; Odds & Bernaerts, 1994; Quindo´s *et al*., 2001).

HiCrome Candida Differential Agar (Himedia, India) is recommended for rapid isolation and identification of *Candida* species from mixed cultures. Perry and Miller (1987) reported that *Candida albicans* produces an enzyme β -N-acetyl- galactosaminidase and according to Rousselle *et al*. (1994) incorporation of chromogenic or fluorogenic hexosaminidase substrates into the growth medium helps in identification of *C. albicans* isolates directly on primary isolation.

Since Polymerase Chain Reaction (PCR) has proven to be a powerful tool in the early diagnosis of several infectious diseases, it might also be a more sensitive alternative assay in the diagnosis of invasive candidiasis. Several PCR methods for the detection of *Candida* spp. in patient materials have been published (Buchman *et al*., 1990; Hopfer *et al*., 1993; Kahn, 1993; Miyakawa *et al*., 1993). The main aim of this study to compare between culture based method (Hicrome Candida Differential Agar) and non-culture based method (PCR) for detection and identification of Candida spp. isolated from intensive care unit (ICU) patients of New Kasr El Aini Medical School Educational Hospital, Egypt.

MATERIALS AND METHODS

1. Sampling and sampling sites:

A total of 309 clinical isolates of Candida spp. were collected from different patient sources; Blood (*n*= 80), Urine (*n*= 120), Sputum (*n*= 73) and Vaginal discharge (*n*= 36) from intensive care unit patients of New Kasr El Aini Medical School Educational Hospital) during the study period (August 2008 to September 2009).

Samples were obtained from patients with clinically proven or suspected systemic Candida infection. The patients' symptoms and characteristics included persistent fever, unresponsiveness to broad-spectrum antibiotic therapy, and specimen positivity by histopathology, etc...

Samples were collected from patients that were isolated in ICU nits for a period not less than 48 h and have not taken any azole drugs.

2. Reference strains:

For quality control, American Type Culture Collection (ATCC) strains were used: *Candida albicans*

ATCC 14053, *Candida glabrata* ATCC 2001, *Candida tropicalis* ATCC 750 and *Candida parapsilosis* ATCC 22019 (MicroBiologics, Saint Cloud, MN, USA).

3. Detection of Candida:

3.1. Culture media:

HiCrome Candida Differential Agar (Himedia, India) with composition: Peptone, special 15.0g/L, Yeast extract 4.0g/L, Dipotassium hydrogen phosphate 1.0g/L, Chromogenic mixture 7.22g/L, Chloramphenicol 0.5g/L, Agar 15.0g/L and 1L Distilled water, was used for rapid isolation and identification of *Candida* species from mixed cultures. 42.72 grams of the dehydrated media was suspended in 1000 ml distilled water and was heated to boiling to completely dissolve the medium. DO NOT AUTOCLAVE. It was cooled to 50°C and pour into sterile Petri plates. The clinical samples (blood, urine, sputum swabs and vaginal swabs) were directly inoculated onto the solidified Hicrome Candida differential Agar using spreading method and incubated at 30°C for 48 hours.

3.2. Non culture method (PCR):

The clinical isolates were kept in Brain Heart Infusion (BHI, 37 g/l; Bio-Rad, Marnes-la-Coquette, France) with 10% glycerol (Vaz Pereira, Lisbon, Portugal), at -70°C. Prior to testing, isolates were retrieved from storage and subcultured, for 24 to 48 h, on plates of Columbia sheep blood agar (bioMérieux, Lyon, France). The PCR technique was carried out according to Huaguo *et al*., (2007) as follow:

3.2.1. Extraction of DNA:

At least one loopful of cells was transferred into an Eppendorf tube containing 400 –800 µl of 1× TE. Then it was heated at 80 °C for 20 min to kill the Candida and then cooled to room temperature.

A 50 µl 10 mg/ml lysozyme was added then, vortex and incubate for at least 1 h at 37 °C. Then add 75 µl 10% Sodium Dodecyl Sulphate (SDS)/ proteinase K solution (5 µl proteinase K, 10 mg/l and 70 µl 10% SDS) (Fermentas), vortex briefly and incubate for 10 min at 65 °C.

A 100 µl 5 M NaCl and 100 µl CTAB / NaCl (4.1 g NaCl, 10 g CTAB in 100 ml distilled water) then was added (Fermentas), which is prewarmed at 65 °C, vortex until the liquid content becomes white (milky), and incubate for 10 min at 65 °C.

Then a 450 µl of chloroform / isoamyl alcohol (24:1) was added, vortex for 10 s, and then centrifuge at room temperature for 5 min at 12,000 g. Transfer the aqueous supernatant to a fresh microcentrifuge tube (Labfuge, 460). Add 450 µl isopropanol, incubate for 10 min on ice, centrifuge for 15 min at room temperature.

Discard the supernatant and wash the pellet with 500 µl of 70% ethanol and centrifuge for approximately 5 min at room temperature. Discard the supernatant and dry the pellet. Redissolve the pellet in 25 µl 1× TE buffer; the DNA can be stored at 4°C until required.

3.2.2. Primer design:

One set of primers targeting the ITS2 region of Candida were used, CN1 with sequence 427 GCATCGATGAAGAACGCAGC 446 and CN2 with sequence 834 TTGATATGCTTAAGTTCAGCGGG T 811. A PCR marker (*Hae*III-digested ΦX174 replicative-form DNA) was used.

3.2.3. PCR amplification:

The reaction mixture (25 µl) contained 2.5 µl of 10× buffer (750 mM Tris–HCl, 200 mM $(NH4)_2SO_4$, 0.1% Tween 20), 2 µl of 10 mM dNTP mixture (2.5 mM eachdNTP), 1.5 µl of 25 mol/L$MgCl_2$, 0.2 µl of each primer (50 pmol), 1 U of Taq DNA

polymerase (MBI America), 5 µl of template DNA, and sterile distilled water to bring the total volume to 25 µl.

PCR amplification conditions were 5 min of denaturation at 95 °C, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, with final extension step of 72 °C for 5 min. Appropriate positive and negative controls were included.

All PCR amplifications were performed by using (BIOER; model TC- 25/H & BIORAD; model PTC 196).

3.2.4. Agarose gel electrophoresis.

Electrophoresis was conducted in TBE (0.1 M Tris, 0.09 M boric acid, 0.001 M EDTA [pH 8.4]) buffer at 76 V for approximately 1 h in gels composed of 1% (wt/vol) agarose (Boehringer Mannheim) and 1% (wt/vol) NuSieve (FMC Bioproducts, Rockland, Maine). Gels were stained with 0.5 mg of ethidium bromide per ml of deionized water for 30 min, followed by a 30-min wash in deionized water. DNA bands confirming a positive PCR were visualized with a UV transilluminator and photographed.

RESULTS

1. Culture method results (HiCrome Candida Differential Agar).

The results in Tables 1, 2, 3 and 4 showed the average counts of Candida spp. isolated from blood, urine, sputum and vaginal swabs samples, respectively using HiCrome Candida Differential Agar in Intensive Care Unit (ICU) of New Kasr El Aini Medical School Educational Hospital.

The obtained results in Table 1 showed that, the average counts of Candida spp. isolated from blood samples were 13.42, 10, 7.71 and 5.57 CFU/ml for *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*, respectively during the sampling period. The minimum counts were 10, 5, 4 and 4 CFU/ml for *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*, respectively. While the maximum counts were 18, 13, 12 and 7 CFU/ml for *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*, respectively. The highest counts of isolated Candida spp. were *Candida albicans* in blood samples, while the lowest counts were *Candida parapsilosis* during the sampling months.

Table 1: Average counts of Candida spp. isolated from blood samples from ICU patients of New Kasr El Aini Hospital (*n* =80).

Candida spp. / Months	Colony forming unit (CFU) / ml			
	Candida albicans	*Candida glabrata*	*Candida tropicalis*	*Candida parapsilosis*
Aug. 2008	14	12	8	6
Sep. 2008	15	12	9	7
Oct. 2008	18	13	12	7
Nov. 2008	16	11	10	6
Dec. 2008	11	10	4	4
Jan. 2009	11	9	6	5
Feb. 2009	13	8	7	6
Mar. 2009	13	5	4	5
Apr. 2009	14	9	9	5
May 2009	12	7	6	6
June 2009	13	11	8	4
July 2009	10	10	8	4
Aug. 2009	13	11	8	6
Sep. 2009	15	12	9	7
Minimum	10	5	4	4
Maximum	18	13	12	7
Average	13.42	10	7.71	5.57

Table 2 showed that, the average counts of Candida spp. isolated from urine samples were 21.14, 14.14, 15.14 and 8.35 CFU/ml for *Candida albicans, Candida*

glabrata, Candida tropicalis and *Candida parapsilosis*, respectively during the sampling period. The minimum counts were 18, 11, 11 and 5 CFU/ml for *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*, respectively. While the maximum counts were 24, 17, 19 and 12 CFU/ml for *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*, respectively. The highest counts of isolated Candida spp. were *Candida albicans* in urine samples, while the lowest counts were *Candida parapsilosis* during the sampling months.

Table2: Average counts of Candida spp. isolated from urine samples from ICU patients of New Kasr El Aini Hospital (*n* =120).

Candida spp. / Months	Colony forming unit (CFU) / ml			
	Candida albicans	*Candida glabrata*	*Candida tropicalis*	*Candida parapsilosis*
Aug. 2008	22	15	17	12
Sep. 2008	23	16	12	9
Oct. 2008	19	14	12	9
Nov. 2008	22	15	18	8
Dec. 2008	20	11	11	7
Jan. 2009	18	12	13	9
Feb. 2009	19	14	14	8
Mar. 2009	22	17	18	10
Apr. 2009	23	15	16	5
May 2009	19	12	13	7
June 2009	20	12	15	7
July 2009	23	14	19	7
Aug. 2009	22	14	17	9
Sep. 2009	24	17	17	10
Minimum	18	11	11	5
Maximum	24	17	19	12
Average	21.14	14.14	15.14	8.35

The obtained results in Table 3 showed that, the average counts of Candida spp. isolated from sputum swabs samples were 23.71, 17.42, 15.21 and 9.14 CFU/ml for *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*, respectively during the sampling period. The minimum counts were 20, 12, 11 and 4 CFU/ml for *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*, respectively. While the maximum counts were 27, 22, 19 and 13 CFU/ml for *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*, respectively. The highest counts of isolated Candida spp. were *Candida albicans* in sputum swabs samples, while the lowest counts were *Candida parapsilosis* during the sampling months.

Table 3: Average counts of Candida spp. isolated from sputum swabs samples from ICU patients of New Kasr El Aini Hospital (*n* =73).

Candida spp. / Months	Colony forming unit (CFU) / ml			
	Candida albicans	*Candida glabrata*	*Candida tropicalis*	*Candida parapsilosis*
Aug. 2008	25	22	19	12
Sep. 2008	26	17	14	12
Oct. 2008	27	19	15	13
Nov. 2008	23	17	15	5
Dec. 2008	20	12	12	8
Jan. 2009	20	14	11	4
Feb. 2009	25	17	14	7
Mar. 2009	20	14	19	12
Apr. 2009	24	17	14	7
May 2009	21	17	14	9
June 2009	24	20	14	5
July 2009	25	18	17	12
Aug. 2009	27	22	17	10
Sep. 2009	25	18	18	12
Minimum	20	12	11	4
Maximum	27	22	19	13
Average	23.71	17.42	15.21	9.14

Table 4 showed that, the average counts of Candida spp. isolated from vaginal swabs samples were 24.85, 19.07, 18.07 and 12.92 CFU/ml for *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*, respectively during the sampling period. The minimum counts were 21, 14, 12 and 9 CFU/ml for *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*, respectively. While the maximum counts were 29, 24, 22 and 17 CFU/ml for *Candida albicans, Candida glabrata, Candida tropicalis* and *Candida parapsilosis*, respectively. The highest counts of isolated Candida spp. were *Candida albicans* in vaginal swabs samples, while the lowest counts were *Candida parapsilosis* during the sampling months. Also, both of *Candida glabrata* and *Candida tropicalis* showed nearly the same averages during the sampling period.

Table 4: Average counts of Candida spp. isolated from vaginal swabs samples from ICU patients of New Kasr El Aini Hospital (*n* =36).

Candida spp. / Months	Colony forming unit (CFU) / ml			
	Candida albicans	*Candida glabrata*	*Candida tropicalis*	*Candida parapsilosis*
Aug. 2008	24	18	12	10
Sep. 2008	25	24	20	11
Oct. 2008	25	14	17	14
Nov. 2008	25	18	17	12
Dec. 2008	24	19	17	13
Jan. 2009	21	17	14	9
Feb. 2009	25	18	17	12
Mar. 2009	22	18	17	13
Apr. 2009	26	19	20	14
May 2009	22	19	19	12
June 2009	27	22	20	15
July 2009	27	21	22	15
Aug. 2009	29	23	22	17
Sep. 2009	26	17	19	14
Minimum	21	14	12	9
Maximum	29	24	22	17
Average	24.85	19.07	18.07	12.92

2. PCR results:

Forty isolates of *Candida albicans*, 20 isolates of *Candida glabrata*, 20 isolates of *Candida tropicalis* and 10 isolates of *Candida parapsilosis* were isolated randomly from HiCrome Candida Differential Agar Petri dishes and examined using PCR technique. From these 40 isolates of *C. albicans*, a fifty isolates the obtained results showed that all isolates of *C. albicans* were positive using PCR (100%).

However, 20 isolates of *Candida glabrata* the results showed that 19 isolates were positive (95%). In addition to that, 20 isolates of *Candida tropicalis* the results showed that 17 isolates were positive (85%).

Moreover, 10 isolates of *Candida parapsilosis* showed that 9 isolates were positive (90%) as showed in Table 5 and Fig. 1.

Table 5: PCR results of Candida spp. isolates from sampling sites during the study period.

Candida albicans		*Candida glabrata*		*Candida tropicalis*		*Candida parapsilosis*	
PCR +	(%)	PCR +	(%)	PCR +	(%)	PCR +	(%)
40/40	100	19/20	95.0	17/20	85.0	9/10	90.0

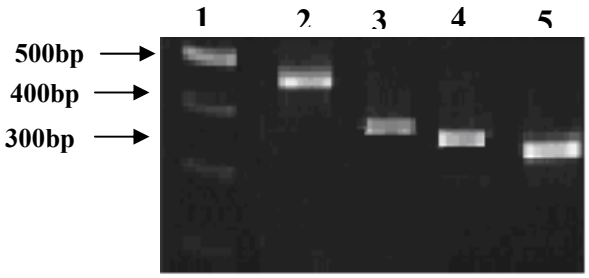

Fig. 1. Agarose gel electrophoresis of the PCR products using the universal primer to amplify the 4 species. Lane 1: marker (*Hae*III-digested ΦX174 replicative-form DNA); Lane 2: *C. glabrata* (450bp); Lane 3: *C. albicans* (380bp); Lane 4: *C. tropicalis* (360bp); Lane 5: *C. parapsilosis* (320bp).

DISCUSSION

Invasive fungal infections represent a major public health concern. In particular, systemic candidiasis remains an increasing source of morbidity and mortality especially in immunocompromised patients such as neutropenic patients undergoing antiblastic chemotherapy or bone marrow transplants (Jarvis, 1995; Pfaller *et al*., 1998; La Valle *et al*., 2000).

Candidemia has been estimated as the fourth most common nosocomial infection with an attributable mortality rate of about 50% (Edmond *et al*., 1999; Gudlaugsson *et al*., 2003).

Candida species may cause severe opportunistic infections, particularly in hospitalized patients. The predominant species remains *C. albicans*, comprising 54-67% of the clinical isolates of Candida (Milde *et al*., 2000; Carvalho *et al*., 2007).

Candida albicans is the most common and clinically relevant pathogen of the genus. However, there has been a significant trend in the emergence of species other than *C. albicans*, with a particular increase in *Candida glabrata* and *Candida krusei* frequency (Pfaller and Diekema, 2002; Tortorano *et al*., 2004) and to a lesser extent, *Candida parapsilosis* and *Candida tropicalis* (Kao *et al*., 1999).

During the study period, we obtained 309 Candida isolates from different body sites (blood, urine, sputum and vaginal swabs) of the hospitalized intensive care and cardiac unit's patients. The urinary tract being the most involved body site among the nosocomial fungal infections, Chen *et al*. (1997), indicated that bloodstream and urinary tract infections were the most frequently seen nosocomial fungal infections.

The results obtained in the study showed that *C. albicans* was the most frequently isolated species in patients with nosocomial fungal infection. It was followed by *C. glabrata*, *C. tropicalis* and *C. parapsilosis*.

These results were similar to those obtained by Findik and Tuncer, (2002) who found that, *C. albicans* was the most frequently isolated species in patients with nosocomial fungal infection (75.4%). It was followed by *C. glabrata* (8.2%), *C. tropicalis* (4.8%), *C. kefyr* (3.4%), *C. parapsilosis* (2.5%), *C. lusitaniae* (1.9%), *C. famata* (1.4%), *C. krusei* (1.4%) and *C. guilliermondii* (1%).

Also, the distribution of Candida species in the present study was similar to the distribution of Candida species reported by Rodero *et al*. (1999); it was *C. albicans* 50.6%, *C. tropicalis* 22.5%, *C. parapsilosis* 20.2%, and *C. krusei* and *C. glabrata* 2.2%.

Chromogenic media such as HiCrome Candida Differential Agar which facilitates rapid isolation of

yeasts from mixed cultures and allows differentiation of *Candida* species namely *C. albicans* (light green), *C. parapsilosis* (off-white to cream), *C. tropicalis* (blue to metallic blue) and *C. glabrata* (cream to white) on the basis of coloration in less than 48 hours.

Although detailed cost-benefit survey were not carried out, it seems clear that these chromogenic media are economical in terms of labor and time. Moreover, their cost would be more than offset by the decreased need for secondary biochemical tests (Baumgartner *et al*., 1996). However, the diagnostic usefulness of PCR remains to be established because of the reported limitations of the technique when whole blood is used (Hopfer *et al*., 1993; Kahn, 1993), the lack of verification with a species-specific probe for the amplified product (Buchman *et al*., 1990), and the inability to detect medically important *Candida* spp. other than *C. albicans* (Miyakawa *et al*., 1992; Van Deventer *et al*., 1995).

The PCR according to its sensitivity and specificity seem promising. The advantages of PCR are the relatively short processing time and its high sensitivity and specificity. The amplification feature of the PCR assay make it ideal for detecting low yeast levels from minimal volume of clinical samples. DNA based diagnosis tests have also the potential to decrease the time taken for the laboratory identification of pathogens that are growing slowly or difficult to culture (Khlif *et al*., 2007).

Moreover, rapid identification of the fungal pathogens such as Candida spp. directly from clinical samples and institution of early therapy may help to reduce the hospital stay and high overall costs associated with management of candidemia. It is also of great value in epidemiological studies.

This study has showed that using of Hichrome Candida Differential Agar was useful for detection and identification of Candida spp. isolated from different clinical samples. The isolated strains were confirmed by PCR technique which showed that most presumptive Candida strains (isolated from Hichrome Candida Differential Agar) were as suspected according to its growth on chromogenic media (Hichrome Candida Differential Agar).

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ARABIC SUMMARY

مقارنة بين الطرق المزرعية و غير المزرعية في اكتشاف العدوى الفطرية المكتسبة داخل المستشفيات في مرضى وحدات العناية الفائقة

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الغرض الاساسى من هذه الدراسة هو المقارنة بين الطرق المزرعية(التى تنمو على Hicrome candida differential agar) و الطرق غير المزرعية بواسطة ال (P.C.R.) و ذلك لتعريف والكشف عن الانواع المختلفة من *Candida species* (*C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis*) المعزولة من مرضى وحدات العناية الفائقة من اكبر خمس مستشفيات تعليمية بالقاهرة مثل القصر العينى الجديد.

وقد أجريت هذه الدراسة خلال الفترة من أغسطس ٢٠٠٨ إلى سبتمبر ٢٠٠٩، و قد اظهرت النتائج ان هناك توافق واضح بين انواع ال Candida المعزولة على الاطباق المحتوية على Hicrome Candida Differential Agar plates) و بين نتائج تحليل ال (P.C.R.) على النحو التالى : ١٠٠، ٠.٩٥ ، ٠.٨٥ و ٠.٩٠ بالنسبة للانواع الاتية :*C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis*, على التوالي. وهذا يعنى أن استخدام (Hicrome Candida Differential Agar media) للكشف عن *candida species*. حساسة للغاية وسريعة واقتصادية الأسلوب