Epidemiological Study of Some *Candida albicans* Strains in Patients Undergoing Chemotherapy by Using PCR Technique in Whole Blood

Ahmad Salih Helal1[1] , Adnan F. AL-azawy[2] , Thekraa Ahmad Hamadaa[3]

[1] *B. Med. Tech./Med. Lab. Sc. North oil company .*
[2] *College of Science Dep. Of Biology , Tikrit university, Tikrit, Iraq .*
[3] *College of medicine , Tikrit university, Tikrit, Iraq .*

Abstract:

Candida species were common human commensals that can cause a wide spectrum of disease, major concern was ahematogenously disseminated infection which was occurring with increased prevalence in immunecompromised patients, although *Candida albicans* remains the most frequent cause of fungemia and hematogenously disseminated candidiasis. In the presented study was 102 patient undergoing chemotherapy and 73 control outpatients returning Tikrit teaching hospital, were examined to determine the prevalence of candidemia by using blood culture method from isolaltion, cultural identification, were 17.6% from cancer patients undergoing chemotherapy positive for candidemia, while all control patient negative. Were83.4% from rural area for province of Tikrit , in addition, were 61.2% from south direction Tikrit city and DNA extraction from whole blood samples dependening on distribution cancer patients and candidemia to detected direct diagnosis and universal strains in our epidemiological study, were 80% *Candida albicans* strains of ATCC 10261, 3153, A72, MEN, Ci002, CiOll, Ci012,CiO35,CiO60, and CiO61 by using PCR technique.

Key: Candidemia, Chemotherapy, Blood Culture, PCR.

Introduction

Opportunistic fungal infections have increased dramatically in recent years. This had often been as a result of advanced medical treatments, such as more intensive regimens of cancer therapy, complications of abdominal or cardiothoracic surgery, prolonged broad spectrum antibiotic therapy, invasive devices such as indwelling catheters and prolonged hospital stays(1,10). Under these conditions an antibiotic-resistant replacement flora, including *Candida* species, can proliferate in the gut and invade deep tissues from mucosal foci. This is especially the case when mucosal integrity has been disrupted due to chemotherapy or surgery. At least 200 fungal species have been identified as human pathogens but most opportunistic fungal infections are caused by yeasts, and the most important yeasts are *Candida* species(3). Rapid identification of *Candida* isolates to the species level in the clinical laboratory has become more important because the incidence of candidiasis continues to rise in proportion to an increasing number of patients at risk for infection with*Cadinda albicans* and recently, with innately azole-resistant non-albicans *Candida* species. Some *Candida* species including *C. glabrata* and *C. krusei* are emerging possibly because they are innately less susceptible to azole drugs (4) . Consequently, rapid identification to the species level is necessary for more rapid and effective antifungal therapy, to facilitate hospital infection control measures and generally because epidemiological studies. Identification of this increasing diversity of pathogen by conventional methods is often difficult and sometimes impossible(5). Morphological features and reproductive structures useful for identifying isolated fungi may take days to weeks to develop in culture, and evaluation of these characteristics requires expertise in mycology. Commercial methods used to identify yeast including API system require 2 to 3 days before biochemical reactions can be interpreted(5). In addition, their databases sometimes are ambiguous. Molecular techniques utilizing amplification of target DNA, provide alternative methods for diagnosis and identification of some organisms(6). PCR based detection of fungal DNA sequences can be rapid, sensitive and specific (7). The positions of the primers used for PCRs within the *C. albicans* rDNA region are shown in Figure 1, synthetic oligonucleotide primers (from DNA Express, Colorado State University) used for amplification of the *C. albicans* NTS fragment were 5'-TAG CGA TGA GGT AGT GCA AGT (PSpA1) and 5'-GCT GCA GCT ACG AAT GTT AG (PSpA2) (8). Using purification of genomic DNA from fresh whole blood collected in EDTA, heparin anticoa-gulant tubes and detected no adverse effects upon subsequent manipulations of the DNA, including PCR(9). Anticoagulant blood samples may be stored at 2–8°C for up to two months, but DNA yield will be reduced with increasing length of storage(9).

The yeast strains used in our study are listed in Table 1. The identities and serotypes of the Candida strains were confirmed with an immune agglutination identification system (Iatron Laboratories, Tokyo, Japan). *C. albicans* A72, MEN, and CiO35 were serotype B; all other C albicans strains were serotype A(8).

TABLE 1. *Candida* strains used

Species	Strain[a]	Source
C. albicans	ATCC 10261	American Type Culture Collection, Rockville, Md.
	3153	National Collection of Pathogenic Fungi, Central Public Health Laboratory, London, United Kingdom
	A72	A. Cassone (Instituto Superiore di Sanita, Rome, Italy)
	MEN	D. Kerridge (University of Cambridge, Cambridge, United Kingdom)
	Ci002, Ci011, Ci012, Ci035, Ci060, and Ci061	Dunedin Public Hospital and School of Dentistry, Dunedin, New Zealand

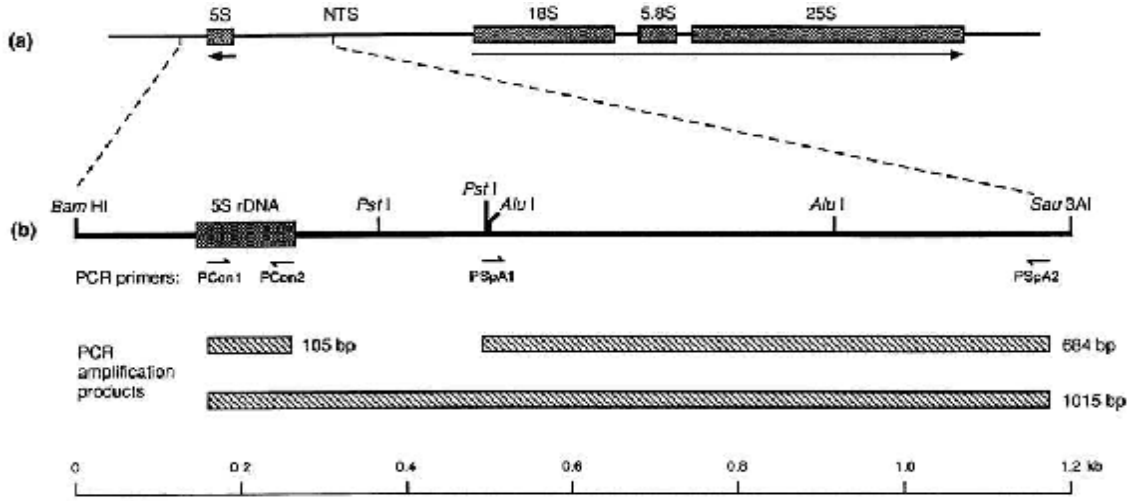

FIG. 1. (a) Physical map of the C. albicans rDNA region showing the positions of rRNA genes, the NTS region, and the direction of transcription of rRNAs (from reference 11). (b) Expanded map of the region showing restriction enzyme sites (from reference 2), the positions of primers used in PCR, and the predicted sizes of the amplified products.

Materials and Methods:-

Patients and control groups :102 patient aged 23-67 years old were undergoing chemotherapy for different types of cancer, and 73 outpatients control group returning in Tikrit Teching Hospital from January 2012 to May 2012.

Specimens : 5ml of venous blood collected from patient and control group; 2.5 ml into EDTA (K3) storage at -32 C^0 for DNA extraction in using for PCR technique and 2.5 ml for yeast blood culture to determine the yeast infection in these two group.

Yeast isolation : slant of 5 ml of brain heart infusion agar overlaid with 20 ml of brain heart infusion broth (Difco) with chloramphincol provided by the Bacteriology Department of the Teaching Tikrit Hospital. were processed for each contained. After inoculation of 2.5 ml of blood into the culture , they were vented and incubated in an upright position for 10 days at 30^0 C. Cultures were examined daily for visual evidence of growth, and each examination was followed by a gentle mixing of the blood-broth mixture over the agar slants. Visual growth was stained and subcultured onto appropriate media for identification. Sabouraud's agar for 48 h at 30°C.

DNA extraction by used Wizard® Genomic DNA Purification Kit (promega) (for 3ml blood sample Volume)[9] and a modification of the hexadecyltrimethyl-ammonium bromide (CTAB) buffer method[12,13].

Procedure.

1- Add 7.5ml of Cell Lysis Solution to a sterile 15ml centrifuge tube.

2- Gently rock the tube of blood until thoroughly mixed; then transfer blood to the tube containing the Cell Lysis Solution. Invert the tube 5–6 times to mix.

3- Incubate the mixture for 10 minutes at room temperature (invert 2–3 times once during the incubation) to lyse the red blood cells Centrifuge at 2,000 ×*g* for 10 minutes at room temperature

4- Remove and discard as much supernatant as possible without disturbing the visible white pellet. Approximately 50–100µl of residual liquid will remain in the 15ml tube .

5-repeat Steps 1–4 until pellet is white. There may be some loss of DNA from frozen samples.

6-Vortex the tube vigorously until the white blood cells are resuspended (10–15seconds).
7- Add700 µl of 2x CTAB buffer (100 mM Tris-HCl [pH 8], 1.4 M aCl, 25 mM EDTA, 2% CTAB),vortex ,transfer to in 1.5-ml microcentrifuge Tubes incubate 60 minutes at $65C^0$. and 700 µl of phenol-chloroform was added. The mixture was Vortexed 5 minute again and centrifuged at 12,000 X g for 5 min. The upper aqueous phase was transferred to a new microcentrifuge tube, and the DNA was precipitated by adding an equal volume of isopropanol and centrifuging at 12,000 X g for 10 min. The DNA pellet was washed once in 70% ethanol [12].
8-. Add 50µl of DNA Rehydration Solution.
9-. Add 1.5µl of RNase Solution to the purified DNA sample. Vortex the sample for 1second. Centrifuge briefly in a microcentrifuge for 5 seconds to collect the liquid and incubate at 37°C for 15 minutes.
10-. Rehydrate the DNA by incubating at 65°C for 1 hour. Periodically mix the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C.
11-. Store the DNA at 2–8°C.

The Used Primers in This Study [14].

No	*Code*	*5⁻ to 3⁻*
1.	**PConl**	**AGT TTC GCG TAT GGT CTC CC**
2.	**PCon2**	**GTT GCG GCC ATA TCT AGC AG**
3.	**PSpA1**	**TAG CGA TGA GGT AGT GCA AGT**
4.	**PSpA2**	**GCT GCA GCT ACG AAT GTT AG**

The reaction mixture 20µL(2µL AccuPower® PCR Pre Mix, 2µL(F,R)primer (1.25 ,uM each), 1µLtemplate 50ng purification and concentration by nanodroup (Thermo®) and 15µL deionizer water). 30 cycles 1:-94ċ-3m. 2:-94ċ-30sc,60ċ-45sc, 72ċ-1m. 3:-72ċ-5m.

Results and discussion

All 102 patients undergoing chemotherapy and control group were tested blood culture classified according to positive and negative blood culture as showed in Table 2. While control group was negative results.

Table 2. prevalence of Candidemia in patients undergoing chemotherapy.

Blood culture results for candidemia	Patients		Controls	
	No.	%	No.	%
Positive	18	17.6	0	0
Negative	84	82.4	73	100
Total	102	100	73	100
X^2 =5.77 P = 0.008 P< 0.01 High significant				

Before discussing studies comparing different techniques for culturing *Candida* spp. from blood, two points regarding their limitations must be made. First, none of these studies describe in detail characteristics of the patients from whom positive cultures were obtained. Thus, it was impossible to determine whether blood culturing was more effective in detecting fungemia in one population of patients compared with another. Frequency of culturing and total volume of blood cultured during an admission might influence the likelihood of detecting fungemia more than the particular blood culture technique used [13,15]. Although there was no similar study in Iraq, particularly in province of Tikrit for comparing this results to other study, this finding give the importance of candidemia in patients undergoing chemotherapy and the significant mortality and morbidity of candidemia associated with this patients that have resulted in considerable efforts to use develop rapid, reliable diagnostic tests, in addition, to use blood culture to facilitate clinical treatment decisions. Despite continued efforts to develop such tests, most tests developed to date lack sensitivity or specificity, the fungal culture were the gold standard for diagnosis [16,15].

The present study revealed that the rate of candidemia in patients undergoing chemotherapy (as shown in table 2) through the isolation of *Candida* spp. from the blood of these patients this finding less than that recorded in other studies; 32.1%[17] in Australia, 32% in USA [18,19], these different results may be due to the difference of techniques for blood culture as the sample size to the volume of media and daily inverting inoculated broth with blood sample. In addition, the method used in our study by BHI broth/agar, that insider as a good significant to screening by visional growth of candid..

The relation between residence group and candidemia revealed that there were 16.6% of infection in urban from total group in South direction 61.2%, but 22.2% in urban from total group 83.4% as shown in table 3. Selected 50% of sampling from each rural, south and other direction to detected (rural 2sample,south 5sample and other direction 3sample) *Candida albicans* strains as showed in table 1, by PCR technique and the prevalence that to region genotyping.

Table 3. prevalence of Candidemia in Residence.

Subjects	Residences						Total	
	Urban		Rural					
			South direction		Other directions			
	No.	%	No.	%	No.	%	No.	%
Patients	18	17.6	70	68.6	14	13.7	102	100
Positive blood culture	3	16.6	11	61.2	4	22.2	18	100
X^2 =19.8 P=0.003 P < 0.01 High significant								

In the presented study the highest rate of increasing candidemia in rural area was 82.4% . Table.3. This may be due to highly contacting with animals that normally found or infected with *Candida* species, In the current other study 75% isolation *Candida* spp. from cow milk[20], poor sanitation and personal hygiene's with low knowledge's about the transmission of Candida from the animals to human. Although the increased rates of candidemia in rural area there is variable of rate within different distribution of locations were the high rates recorded within rural areas of south direction from Tikrit provinces, this may due to environmental factors or type of animal domestication that harbors *Candida* species that affecting the spreading the infection.

PCR technique results:-

Table 4. Nanodruop Results.

data	No. of sample	DNA concentration ng/µL	purfication A260/A280
14/05/12	1	81.3	1.27
14/05/12	2	120.5	1.88
14/05/12	3	91.9	2
14/05/12	4	66.1	1.9
14/05/12	5	158.9	1.58
14/05/12	6	61.3	2
14/05/12	7	33.7	1.09
14/05/12	8	85.4	1.91
14/05/12	9	50.2	1.87
14/05/12	10	68.3	1.9

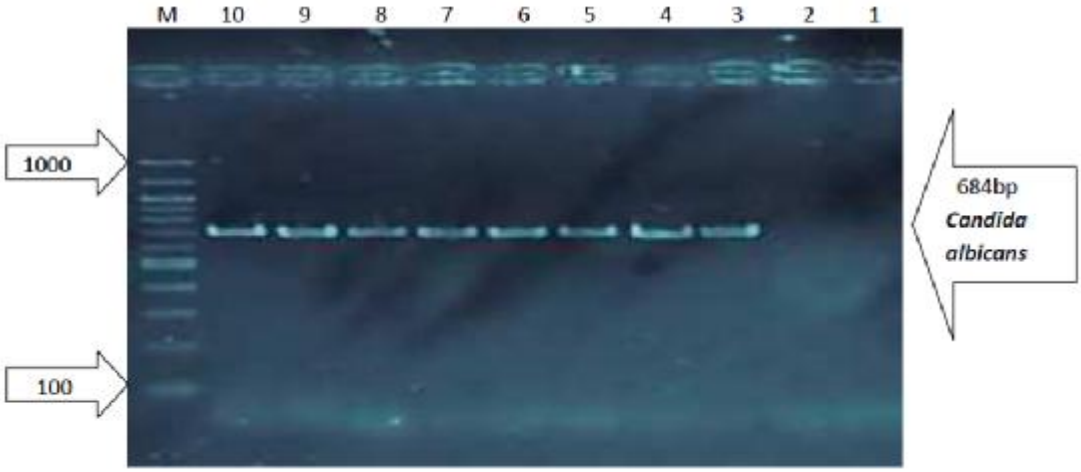

Figure 3. Electrophoresis 2% agarose jel within 1X TBE at stage 1 V.45,mA 60,T.15 and stage 2 V.60 ,mA 90,T.120

The idea of epidemiological strain study beyond the diagnosis of the colony identification to reduce the cost and to demonstrate the level of strains in the pathogen were selected samples of whole blood of a disease in our study, there were 5 samples isolated from rural south direction, 3 rural other direction and

2 urban residence as shown in table 3, completed 10 sample whole blood from all residence patients cancer undergoing chemotherapy and camdidemia was detected by PCR Technique, were 80% *Candida albicans* results and 20% non-albicans as show in Figures 3.

The epidemiological strain study by rapid diagnosis of *Candida* species through DNA extraction of *Candida* from fresh blood sample of patients undergoing chemotherapy were 80% for *Candida albicans* while 20% for non albicans candida. This agree with geographic distribution of candida[21].

Identification of candidemia takes a minimum of 2 days with an optimal blood culture system[9], so there was a need for a rapid, sensitive, and specific test to aid in diagnosis of disseminated yeast infection. DNA- diagnostic methods not sensitive and specific only but also, have the potential decrease time taken for the laboratory identification of pathogens that were slowly growing or difficult to culture. Such tests may detect nonviable and non cultureable cells as well as viable cells. Drug susceptibilities of Candida species vary; for example, most C. krusei strains are resistant to fluconazole[22]. Therefore, earlier detection and identification of the infecting species in blood would facilitate prompt, appropriate treatment.

In conclusion, the PCR assay designed and tested as described here provides a high sensitivity and specificity for the detection of fungal DNA in blood samples and rapidly identifies most *Candida* species. Thus, the vast majority of clinically relevant fungal pathogens in the immunocompromised patient were detected by this assay, distribution of *Candida albicans* in patients undergoing chemotherapy was a high rate and Nan-albicans Candida as a significant rate. BHI agar with broth for isolation was increased visual growth screening, using IgG and IgM antibody by ELISA technique increased incidence of early detection candidemia, CTAB buffer as instead lyticase enzyme in promega extraction DNA kit, in additional, incubation the buffer at 65^0C in 60 minutes was good methods, PCR technique were very good methods for diagnosis candidemia in patients undergoing chemotherapy, The strains of *Candida albicans* detection by PCR was same strains in USA, Italy, UK and New Zealand.

Refeence

1. **Wingard JR**. Importance of Candida species other than *Candida albicans* as pathogens in oncology patients. *J Clinical Infect Dis* **1995**; 20: 115-125.

2. **Cannon R D, Jenkinson H F, Shepherd M G.** Isolation and nucleotide sequence of an autonomously replicating sequence (ARS) element functional in *Candida albicans* and Saccharomyces cerevisiae. J Mol. Gen. Genet. **1990**. 221:210-218.

3. **Elie CM, Lott TJ, Reissn E, Morrison CJ**. Rapid identification of Candida species with species specific DNA probes. *J Clin Microbiol*. **1998**; 36: 3260-3265.

4. **Shin JH., Notle FS, Morrison CJ**. Rapid identification of candida species in blood cultures by a clinically useful PCR method. *J Clin Microbiol*. **1997**;35: 1454-1459.

5. **Bchman T, Rosier GM, Merz EG, Charache P**. Detection of surgical pathogens by in vitro DNA amplification. Part one. Rapid identification of *candida albicans* by in vitro amplification of a fungus-speceific gene. *Surgery* **1990**; 108: 308-374.

6. **Burgener-kairuz Pzuber JP, Jaunin P, Buchman TG, Billie J, Rossier M.** Rapid detection and identification of *candida albicans* and *torulopsis* (candida) *glabrata* in clinical specimens by species-specific nested PCR amplification of cytochrome P-450 lanosterol a demethylase (L1 A1) gene fragment. *J Clin Microbiol.* **1994**; 32: 1902-1907.

7. **Campin AC, Matthews RC**. Application of the polymerase chain reaction to the diagnosis of candidosis by amplification of an HSP 90 gene fragment. *J Med Microbiol*. **1993**; 39: 233-238.

8- **Holmes A R**, **Cannon R D, Jenkinson H F, Shepherd M G**. Detection of *Candida albicans* and Other Yeasts in Blood by PCR. *J. Clin. Microbiol.* 1994, 32(1):228.

9. **Technical Manual, Wizard® Genomic DNA Purification Kit,** Instructions for use of products A1120, A1123, A1125. Available from **www.promega.com.**

10-. **Pfaller MA**. Nosocomial fungal infections ;epidemiology of candidiasis. *J Hospital Infec* **1995**;30 (Suppl.): 329-338.

11. Magee, B. B., D'Souza T. M., and Magee P T. Strain and species identification by restriction fragment length polymorphisms in the ribosomal DNA repeat of Candida species. *J. Bacteriol*. **1987**. 169:1639-1643.

12- **O'Donnell K and Gray L E**.. Phylogenetic relationships of the soybean sudden death syndrome pathogen *Fusarium solani f* sp. *phaseoli* inferred from rDNA sequence data and PCR primers for its identification. *J Mol. Plant-Microbe Interact*. **1995**. 8:709-716.

13- **Murray P R, Baron E J,. Pfaller M A, Tenover F C, and Yolken R H.**. Manual of clinical microbiology, 6th ed., p. 699-737. ASM Press, Washington, D.C. 1995.

14. **Alpha DNA** source of shopping primers in our study. Available from http://www.alphadna.com.

15. **Jeffrey M. Jones**. Laboratory Diagnosis of Invasive Candidiasis. *J Clin Microbiol*.. **1990**;(3):32-45.

16. **Jabra-Rizk MA, Falkler WA, Meiller TF**. Fungal biofilms and drug resistance. J *Emerg Infect Dis* **2004** Jan;10(1):14-9.

17. **Chen S, Slavin M, Nguyen Q, *et al***. Active surveillance for candidemia, Australia. *J Emerg Infect Dis* **2006** Oct;12(10):1508-16.

18**.Theoklis E Z, Jesse A.** The Epidemiology and Attributable Outcomes of Candidemia in Adults and

Children Hospitalized in the United States: A Propensity Analysis. Philadelphia. *J Clinical Infectious Diseases***. 2005**;41:1232–9.
19. **Diekema DJ, Messer SA, Brueggemnaa AB. *et al***. Epidemiology of candidemia: 3-year results from the emerging infections and the epidemiology of Iowa organisms study. *J Clin Microbiol*. **2002**; 40: 1298–302.
20**. Tarfarosh M A, KPurohit S.** Isolation of Candida spp. from Mastitic cows and Milkers. Vetscan - *Online Veterinary Journal*. **2008**;. 3: 28.
21. **Pfaller MA, Diekema DJ**. Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of Candida. *Clin Microbiol Infect* **2004** Mar;10 Suppl 1:11-23.
22. **Ness M J, Vaughn W P, Woods G L**. Candida antigen latex test for detection of invasive candidiasis in immunocompromised patients. *J.Infect. Dis.* **1989**;159:495-501.

احمد صالح هلال[1] ، عدنان فاضل العزاوي[2] ، ذكرى احمد حمادة[3]

[1] شركة نفط الشمال ، العراق

[2] كلية العلوم، جامعة تكريت ، تكريت ، العراق

[3] كلية الطب ، جامعة تكريت ، العراق

الملخص

أجناس خمائر المبيضة الاكثر تعايشا للأنسان والتي تسبب العديد من ألأمراض الواسعة الانتشار وخاصة المرضى غير السوي مناعيا .والقلق البالغ من تداخل وانتشار الأصابة والتي تظهر ضراوة عالية في مرضى غير السوي مناعيا.وان المبيضة البيضاء الاكثر اصابة من بين اصناف المبيضات. اجريت الدراسة على 102 مريضا خاضعين للعلاج الكيميائي والمصابين بمختلف انواع السرطان، و73 مريضا مراجع للمستشفى كمجموعة سيطرة واللذين ارتادو الى مستشفى تكريت التعليمي خلال الفترة من كانون الثاني 2012 الى ايار 2012 .اظهرت الدراسة والتي اجريت على نماذج الدم للمرضى لعزل الخمائر المبيضة باستخدام بعض الاوساط الزرعية الخاصة و تشخيصها ، وجد 17.6% من المبيضات في الدم ولم تجد أي أصابة في مجموعة السيطرة، وأن 83.4% من الأصابة هم من المناطق الريفية للمحافظة وأضافتا لذالك 61.2% من الأتجاه الجنوبي للمدينة تكريت واستخلاص الحمض النووي من نماذج الدم العشرة أعتمادا على التوزيع وأنتشار مرضى السرطان المصابين للتأكيد التشخيص السريع و للتعرف سلالات المبيضة البيضاء كانت من ضمن السلالات الشائعة عالميا باستخدام تقنية بلمرة التفاعل المتسلسل.