Journal of Natural Sciences Research www.iiste.org
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.4, No.5, 2014

Detection of ERG11-2 gene *in candida spp.* wich resistant to some antifungal agents by Real Time –PCR

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Abstract:
The present study was aimed to determine ERG11-2 that responsible of resistance to some antifungal agents in *candida spp*. Atotal of(107) samples were obtained fromdifferent clinical cases included(36) oral swabs from children suffering from oral thrush , (32) vaginal swabs from women suffering from vaginal candidiasis ,(39) urine samples from individuals suffering from urinary tract infection) and(25) samples from sewage water as a control samples in the molecular study .The result of morphological and biochemical tests showed For pathogenic samples that 83(77.5%) isolate of *candida spp*. Included 36/31(86.1%) from oral samples,32/28 (75%) from vaginal swabs, 28/39 isolates (71.7%) from urine samples while the result of non- pathogenic samples revealed that 20 (80%) isolate of candida spp. Detecting of,ERG11-2 resistant gene was done by RealTime-PCR technique . Results showed that all isolates 31 (100%) were carrying ERG11-2 that responsibe of resistance in candida to some antifungal , Did not record any isolated from control samples from sewage water wich amounted to (9) the ERG11-2resistantgene
Key words: ERG11gene , candida sp., RT-PCR

Introduction:
Increased in recent patterns of resistance to treatment are unacceptable, especially in patients with malignant tumors and organ transplants where injecting with antibiotics broad-spectrum and taking intravenous fluids and there are several mechanisms to explain drug resistance to the antibiotic of these mechanisms is that the pathgenetic species such as fungi are inherently resistant before exposure to the antibiotic due to the lack of ownership of the metabolic pathway or metabolic component, which lies upon the mechanism of action of antibiotic(Marie &White ,2010)

Gaining fungi resistance by several mechanisms, including genetic mutations, which are often fixed mutations which transmitted from one generation to another ,there are families of genes unique to Candida these include genes encoded for enzymes and genes related to adhesins and genes related tosuperoxid dismutases resistance in addition to the genes called resistance genes such as ERG11,CDR1,CDR2(Manoharlal.,2008)

These genes are prone to genetic mutations and these mutations play a role in increasing the gene expression of the resistance and these mutations are called point mutation, for example, ERG11gene that present inpathogenic species of Candida treatment with azole compounds have a point mutation.in it (Tavakoli et.ai.,2010)

Therefore, this study aimed to detect and identify point mutation that occurs in some of the genes responsible for gene expression of resistance to antifungal in some species of candida using RT- PCR technique

Materials and methods:

Samples collection:

Collection of 107 clinical specimens from hospital women and children, (36 oral swab of children are infected with oralthersh 0.32 vaginal swab from women with vaginal candidiasis and urinary sample of 39 person is infected with the urinary tract). During the period from October 2012 - to March 2013.

Isolation and identification

Cultured samples on Sabouraud's Dextrose Agar, streaking smear cotton on the surface of the center of media, for samples of urian tract was cultuered by using (sterile Loop)) each sample worked three replicates , the dishes were incubated at 37 C ° for 24 hours (Atlas, 1995)).

Biochemical tests

Sugars fermentation test:

Examination was conducted based on the method of (Robert, 1990).

Sugars assimilation test:

Examination was conducted based on the method of (Bukly, 1989)).

Urease Enzyme Production:

Examination was conducted using the tapes API-Candida

Molecular diagnosis:

Table(1)Primer of DNA used in this work

primer	Sequence($3^{/}$-$5^{/}$)		Target size	source
ERG11-2	GTT GAA ACT GTC ATT GAT GG	F	96bp	(Victoria et al., 2012)
	TCA GAA CAC TGA ATC GAA AG	R		

Genomic DNA extraction:

DNA was extracted from *Candida spp*. By used Bio Basic kit and according of instructed by the company processed.

Real-Time PCR master mix:

Reaction mixture was prepared Real-Time PCR using several of the AccuPower ® 2X GreenStarTM qPCR Master Mix supplied by the company Bioneer Korean and as instructed by the company.

Analysis of real-time PCR results:

Has been analyzing the results of examination of the Real-Time PCR through turn inflation Amplification plot based on number line voltage threshold Threshold cycler number (CT) value, since the sample is positive when it exceeds that threshold , and also have been identified privacy examination of samples positive by curve fusion melt curve, as the samples are positive if they appear in the same melting point ranging in real inflation of the gene of Tm 80-90 ° C. Can be detected easily and reliably from the privacy of interaction through the analysis of curved fusion Melting curve analysis of the output of the process after PCR amplification reactions .

Result &Discusion :

Diagnosed 83 isolates (77.5 %) by isolating total of (107 samples) was isolated and ***Cabdida Spp*.:** diagnosed with four types belonging to the genus *Candida spp*. isolated from different clinical samples included vagina , mouth and , , as shown in Table (2) .

The results showed that the percentage of *C.albicans* accounted for the largest proportion of species isolated from clinical specimens(table 3) this result accordenat with (Al-Obady, 2012) as isolated five species belonging to the genus *Candida* from the oral cavity , vagina, and came species *C . albicans* in the first, as well as accordenat with (Hussain, 2011). isolate five species as belonging to the genus *Candida* from the oral cavity and record species *C. albicans* higher percentage and was followed by *C. parapsilosis, C. krusei, C. tropicalis* ..

Twenty samples have been diagnosed with isolation (80% of the total 25 samples) was isolated and diagnosed four species belonging to the genus *Candida spp. .Candida* in the current study, based on biochemical characteristics, as isolated from sewage and taken to compare with the species to molecular study (table 4).

Tested of antifungal susceptibility showed isolates of *Candida* under study have variation in resistance to the antibiotics used , as shown isolates of yeasts high resistance towards fluconazole and gresiofulvin as the percentage of resistance (100%) for, gresiofulvin, % 84.3for fluconazole either counter clotrimazole came resistance ratio (73.4%) , and Ketoconazole has shown isolates resistant towards him by (25.3 %) As for the nystatin results have shown that it is best options therapeutic as percentages of resistance(12)%) , table (5)

The results pointed to the relative resistance of most yeast isolates under study for most of the antibiotics used , nystatin of the most influential antifungal- emergence of the highest areas of inhibition ., this is due to the nystatin works to discourage the manufacture Ergosterol important in the construction of the cell membrane of yeast .This result is accordenat with (Al-Shibli , 2006) which found that Nystatin was one of the best antibiotics in growth inhibition *Candida* . The Antifungal Ketoconazole have inhibition zone (32_22) mm had a clear impact damped where 62% of the species of yeasts isolated , and this is accordenat with(Moussawi ,2003) as increased sensitivity to its Isolates with Ketoconazole increase the concentration of the counter . The Clotrimazole ranked third in terms of the ability to inhibit the growth of *Candida* species this explain by Abdul-Hussein (2001) that this counter- efficient in inhibiting the growth of Candida . ,(Fluconazole) came in fourth place in the inhibition of growth of Candida species under study where it was percentages of inhibition of 15.6 %, and this result may be due to the large number of use of antibiotics , as well as the evolution of the type of resistance possessed by these isolates against most of the antibiotics used (Sibanda and Okoh, 2007), and finally Grisofulvinranked the last in the inhibition of growth of Candida species under study where the rates of inhibition 100% this finding by (Shibli , 2006) , where the Griseofulvin did not show any effect on the yeast candida did not show inhibition of growth within the concentrations used and thus coincided our results with the findings of the(Myrvik, 1988; Al-Moussawi , 2006) which found this counter has no influence on the yeast candida

The isolates were resistance to antifungal sure they contain a gene ERG11-2 (resistant to some antibiotics innate : FLC, CC, KT) using the technique (PCR RT-), as it showed the (31) isolates contain this gene by 100 %) (in pathogenic isolate, and therefore promised these isolates were resistant to the azoles . longer gene ERG11-2 is responsible for the phenomenon of resistance to treatment as a result of the boom raster the point mutation leading to an increase in gene expression him over expression of the qualities diagnostic isolates resistance , where the results of the investigation of the gene using a technique RT-PCR to own a isolates , which was conducted by screening , 31 isolates (positive for roads previous) by (100%) of the gene ERG11-2 as shown in the table (6) , and do not note this gene in isolates control the amount of (9) isolated as described in forms a, B): 1), (2: A, B), (3, A, B), (4, A, B) where the ownership is Pathogenic isolates for gene ERG11-2 in the current study to study 0f(Victoria (2012) for molecular analysis using the technique conventional-PCR approach to our results as identified mutation raster gene resistance at the molecular weight bp 92 of the species of *candida parapsilosis, candida parasilosis, candida albicans* resistance to anti- azole and 100% when tested for certain species of candida enabled PCR to make sure they contain a resistance gene mentioned above. And the ratio of the distribution of the gene in our study are as follows (samples pathogenesis and control samples that have been investigated technology RT-PCR was 40 isolation rate (77.5%) and by 13 (32.5%) isolated from the vagina , and 11 (27.5 %) isolates of lactation , and 7, 17.5) %) isolated from the mouth for the sick and samples (22.5 %) 9 isolation of unsatisfactory samples) as shown in the table (6,7) .

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Table(2):number and percent of isolationof *candida sp.* From different samples

Clinical samples	No.of tested samples	No. of +ve samples	%	No. of -ve samples	%
Oral thrash	36	31	86.1	5	13.8
Vaginal swabs	32	24	75	8	25
Urine	39	28	71.1	11	28.2
Total	107				
$X^2_{cal.}=0.266$ $X^2_{tab.}=2.64$	p<0.05*significant				

Table(3) number and percent of species of *candida*

species	Vaginal swabs	%	Urine samples	%	Oral samples	%
C.albicans	9	37.5	14	50	16	51.6
c.tropicalis	7	29.1	10	35.7	6	19.3
C.parapsilosis	5	20.8	3	10.7	5	16.1
c.krusei	3	12.5	1	3.6	4	12.9
total	24	100	28	100	31	100
LSD=7.4 between isolates for each sample LSD=6.34 between samples for each isolate Interference= *significant p< 0.05						

Table(4) percentage of *candida sp*. That isolation from

Controle samples	No. of tesed samples	No. of +ve samples	%	No. of -ve samples	%	Total	%
	25	20	80	5	20	25	100
X^2_{tab}= 0.43 $X^2_{cal.}$=320.56 p<0.05*significant							

Table(5) Diameter of inhibition zones of candida sp. Toward different antifungal

Antifungal Inhibition zone diameter(mm)		Resistance		sensitive	
		No.	%	No.	%
NS	30-17	10	12	73	87.9
KT	22-16	21	25.5	62	74.6
CC	19-15	61	73.4	22	26.5
FLC	10-9	70	84.3	13	15.6
GRE	5-0	83	100	0	0

NS=Nystatin ,KT=Ketoconazole , CC= ,FLC=Fluconazole
GRE=Gresiofulvin

Table (6) No.and percentage of ERG11-2 in pathogenic *Candid* andnon pathogenic *candid*a by PCR-RT

Resistant genes	No.of pathogenic candida	%	No.non pathogenic candida	%
ERG11-2	31	100	9	100

Table(7)No.and percentage of ERG11-2 according of source of isolation

Genes	Vaginal swabs	Urine samples	Oral samples	Nonpathogenic candida	total
ERG11-2	13(32.5%)	11(27.5%)	7(17.5%)	9(22.5%)	40(77.5%)

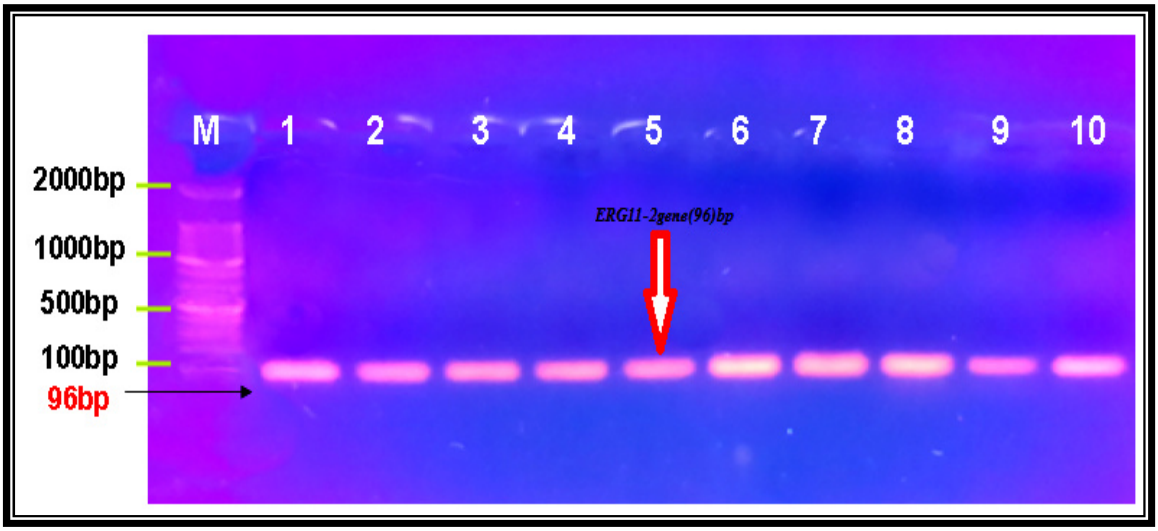

Figure: A): 1): gel electrophoresis that the results of polymerase chain reaction to the point mutation of the gene ERG11-2-resistant anti-fungal using PCR RT-. Where M = DNA Ladder marker (100-2000bp), pathogenic isolates showed a positive result for gene ERG11-2 is responsible for increasing exprresion of gene to resistance to antibiotics KT, FLC, CC. Samples of (1-4) for *Candida tropicalis*, samples (5,6,7,.10) *for C.albicans*, samples (8.9) for *C.krusei*

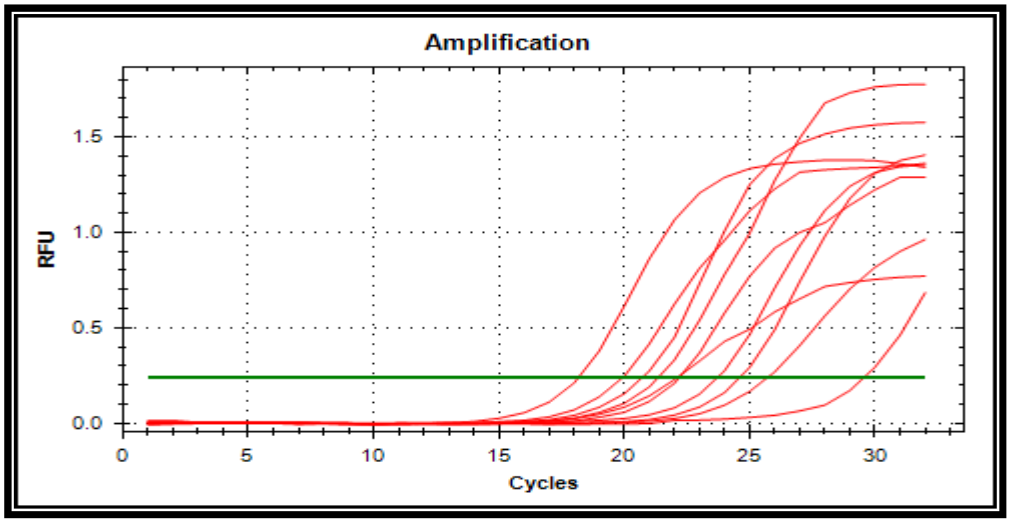

Figure: B): 1) the inflation curve (RT-PCR) as explained point mutation of the gene ERG11-2for samples above.

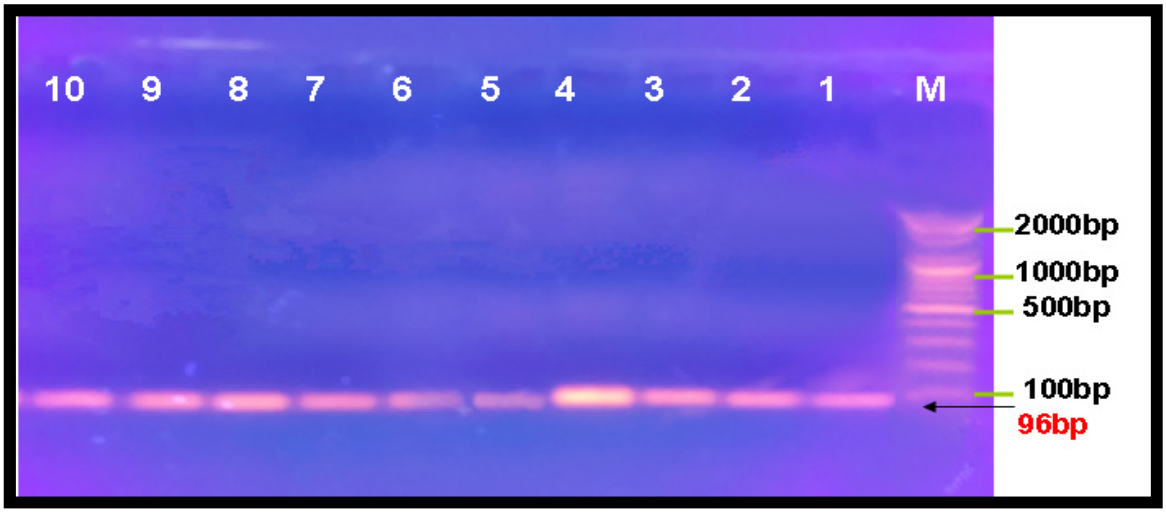

Figure (A: 2) gel electrophoresis that the results of the polymerase chain reaction to the boom raster point mutation of the gene ERG11-2-resistant anti-fungal using PCR RT-. Where M = DNA Ladder marker (100-2000bp), pathogenic isolates showed a positive result for geneERG11-2 is responsible for increasing exprresion of gene to resistance to antibiotics KT, FLC, CC. Sample (6) for *Candida tropicalis*, samples (1-5.8 ,.9) for *C.albicans*, the sample (10) *for C.krusei* and the sample (7) for *C.parapsilosis*.

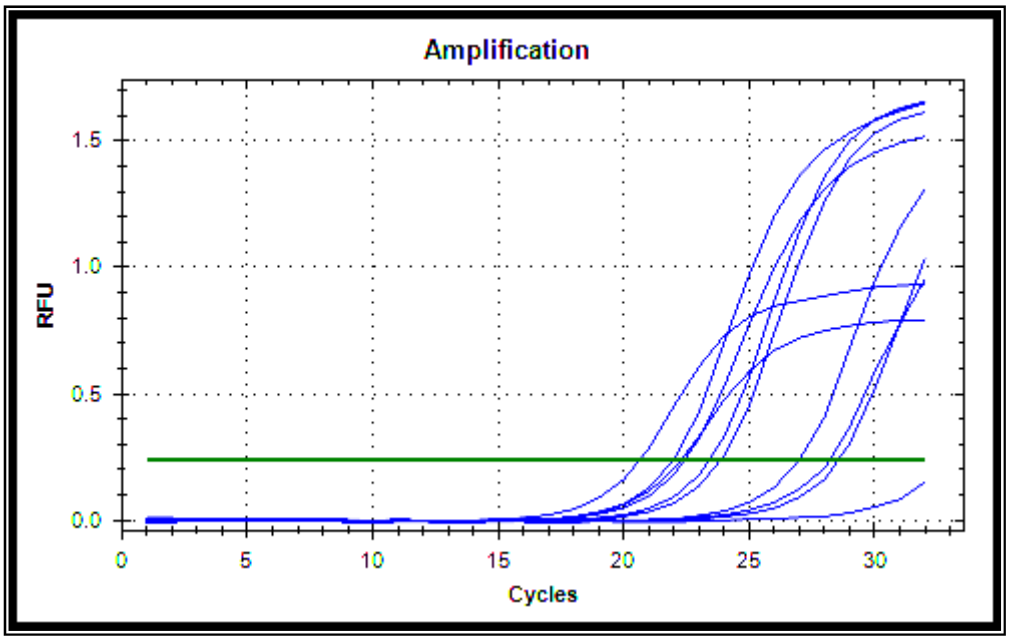

Figure (B: 2) the inflation curve (RT-PCR) as explained point mutation of the gene ERG11-for samples above.

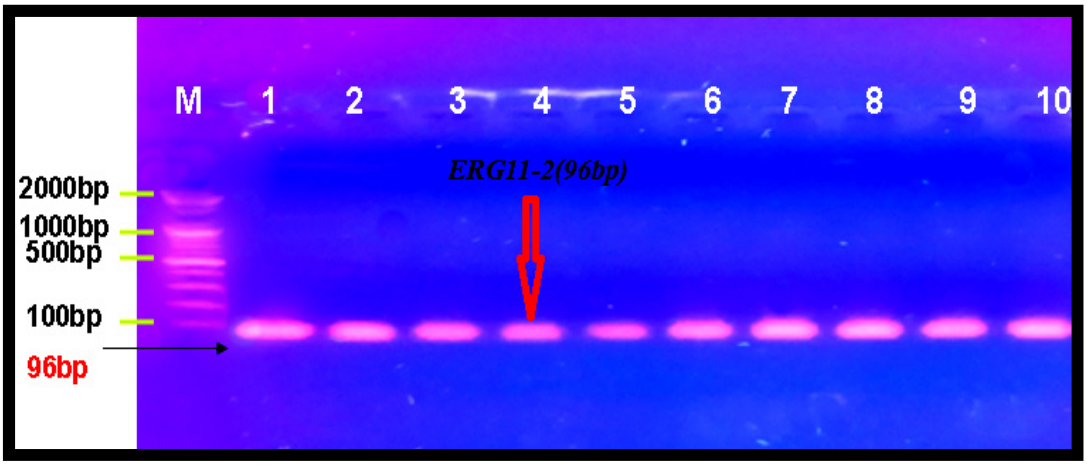

Figure (A: 3) gel electrophoresis that the results of the polymerase chain reaction to the boom raster point mutation of the gene ERG11-2-resistant anti-fungal using PCR RT-. Where M = DNA Ladder marker (100-2000bp), pathogenic isolates showed a positive result for gene ERG11-2 is responsible for increasing exprresion of gene to resistance to antibiotics KT, FLC, CC. The sample (6.3) for *Candida tropicalis*, samples 1,2,5,7,9,10)) for *candida albicans* the sample (8.4)for *C.kruse*

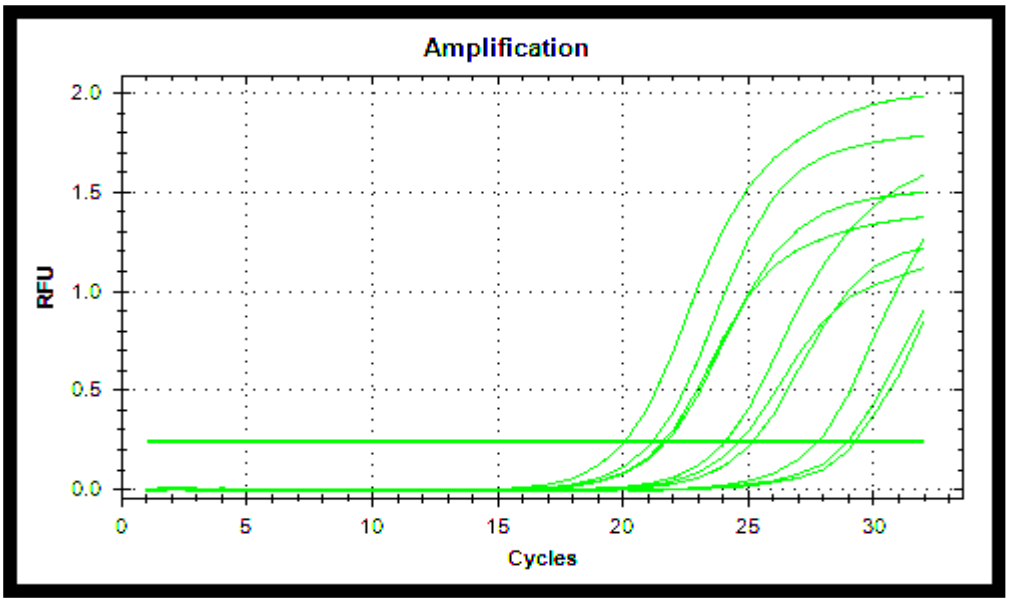

Figure: (B: 3) the inflation curve (RT-PCR) point mutation of the gene ERG11-2 of the samples above.

Figure (A: 4) that the results of the examination of the polymerase chain reaction to the boom raster point mutation of the gene ERG11-2-resistant anti-fungal using PCR RT-. Where M = DNA Ladder marker (100-2000bp), pathgenic isolates showed a positive result for gene ERG11-2 is responsible for increasing exprresion resistance to antibiotics KT, FLC, CC. Sample (1) for *C.albicans* isolation to control (10-2) nonpathogenic isolates showed a negative result for the absence of the gene.

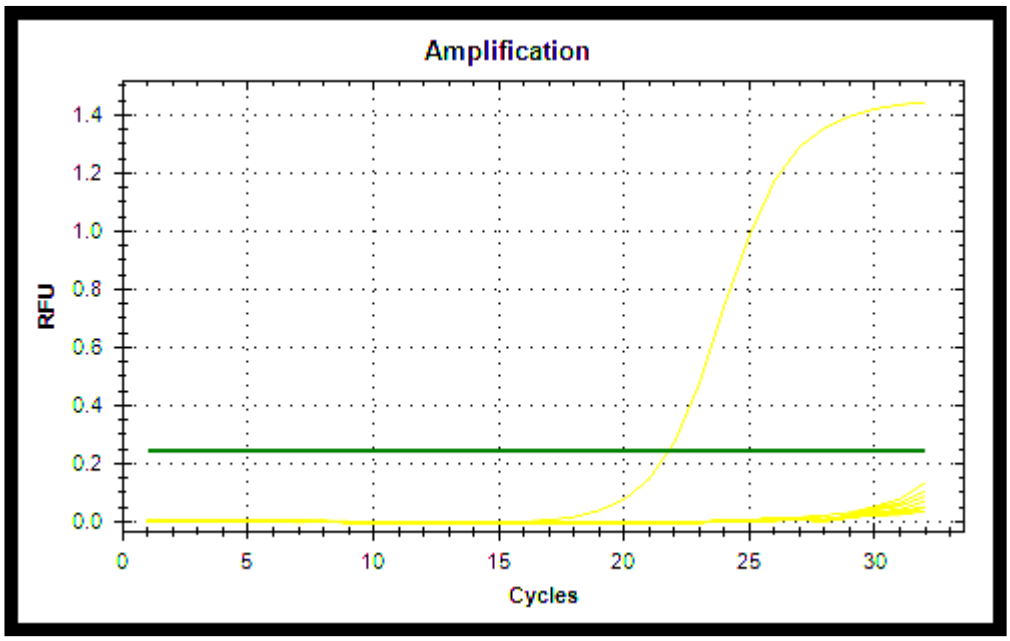

Figure: B, (4 the inflation curve (RT-PCR) amplification, as is the initiator in the device during interaction with the stian (SYBRGreendye) samples nonpathogenic with a control sample represented of yellow line passing through the Threshold line)).