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Detection of CYBB Gene Expression by Reverse Transcription PCR as a Diagnostic Tool for X-Linked Chronic Granulomatous Disease in Egyptian Children

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Abstract

Background: Chronic granulomatous disease (CGD) is a genetic syndrome characterized by a dysfunction of the respiratory burst, which is necessary to kill certain phagocytosed pathogens. The fundamental defect in CGD lies in the NADPH oxidase, the enzyme complex responsible for initiating the respiratory burst. Defects in any of the six subunits of the NADPH oxidase enzyme can manifest as CGD. Thus, CGD patients can be phenotypically similar but genetically heterogeneous depending on which NADPH oxidase component is defective. By far the most common form of CGD is the X-linked recessive form. It results from a mutation of the CYBB gene encoding for gp91phox subunit of NADPH oxidase resulting in a greater number of affected males. However, there have been reports of affected females with the diagnosis of X-linked CGD attributed to skewed X chromosome inactivation.

Aim of Study: In the present study we aimed to diagnose the X-linked type of CGD in a group of Egyptian children by detection of CYBB gene expression using real time RT-PCR and to investigate if it correlates with the test of phagocytic lytic index as a cheaper diagnostic method for CGD.

Patients and Method: This case-control study was conducted on 15 provisionally diagnosed CGD patients (Group I) by the use of DHR test, with the stimulation index using PMA <30%. They were recruited from different university hospitals in Egypt. The study also included 12 mothers (Group II) of the studied patients to detect the genetic mutations in carriers, if any, and 14 apparently healthy children as a control group (Group III).

Results: We found that cases with a fold change of CYBB gene expression less than 0.34 (cut-off value calculated by the 25th percentile fold change of the control group), are considered having defective CYBB gene expression. At this cut-off value, 3 males (20% of all cases in group I and 33% of the males in group I) showed under-expression of the CYBB gene, while none of the females in this group showed defective CYBB gene expression. Also, no mother in group (II) showed under-expression of CYBB gene at that cutoff value. The diagnostic characteristics of fold change of CYBB gene expression by real-time RT-PCR technique at the cut-off 0.34 showed sensitivity = 20%, specificity = 86%, PPV = 60%, NPV = 50% and test accuracy = 52%.

Conclusion: We could establish the diagnosis of 3 out of 15 CGD cases as X-linked form, derives from defects in the CYBB gene, which encodes gp91phox of the oxidase, without the need to use complex and expensive methodologies such as northern blot, slot blot, or genomic DNA sequencing. Despite the low number of patients included in this study to draw definite conclusions, this molecular analysis provides an insight into the breadth and relative distribution of genetic abnormalities responsible for the disease. Overall, we have demonstrated that RT-PCR, a simple and a relatively low cost methodology in comparison to other complex molecular diagnostic methods, may be a suitable tool for diagnosing CGD in laboratories in developing countries.

Key Words: *CGD – CYBB – NADPH – PID.*

Introduction

PIDs are a group of genetic defects characterized by abnormalities of one or more components of the immune system. While there have been several advances in diagnosis, management, and research in the field of PIDs, they continue to remain underdiagnosed, especially in the less affluent countries. More than 300 genetically defined single-gene inborn errors of immunity are now recognized as a cause of PID **[1].**

CGD is an inherited disorder of the innate immune system characterized by a defective oxidative burst of phagocytes and subsequent impairment of

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their microbicidal activity. Mutations in one of the NADPH oxidase components affect gene expression or function of this system, leading to the phenotype of CGD [2]. NADPH Oxidase enzyme consists of six different subunits that interact to form an active enzyme complex responsible for the production of ROS. In neutrophil, the large membrane-bound heterodimeric cytochrome b558 (cyt. b558) is comprising a large glycosylated subunit gp91phox (also known as the β subunit) and a small non-glycosylated subunit, p22phox (also known as the α subunit) [3]. The cytosolic components of NADPH oxidase include p40phox, p47phox and p67phox. P47phox is phosphorylated in stimulated neutrophils, which increases the binding of this component to p67phox and Rac approximately 100- and 50-fold, respectively, and these interactions lead to the translocation of the cytosolic complex to the membrane [4].

Many mutations have been identified the in the CYBB gene responsible for X-linked CGD. Sequencing of all exons and intron boundary regions revealed mutations. The types of mutations included large and small deletions (11%), frame shifts (24%), nonsense mutations (23%), missense mutations (23%), splice-region mutations (17%), and regulatory-region mutations (2%). The distribution of mutations within the CYBB gene exhibited great heterogeneity, with no apparent mutational hotspots. The heterogeneity of mutations and the lack of any predominant genotype indicate that the disease represents many different mutational events, without a founder effect, as is expected for a disorder with a previously lethal phenotype [5].

The X-linked recessive form of CGD is by far the most common resulting in a greater number of affected males. It accounts for 65% to 70% of CGD cases in Western countries and those patients have the most severe phenotype [6]. However, there have been reports of affected females with the diagnosis of X-linked CGD attributed to skewed X-inactivation according to the "Lyon Hypothesis" of X-chromosome inactivation. Generally this is a random process of inactivation with an expected ratio of 50/50, although in reality the ratio follows a Gaussian distribution [7]. Lyon hypothesis assumed that; female subjects have 2 populations of neutrophils: one set expressing the paternal X-chromosome and the other set expressing the maternal one. In female mammals most genes on one randomly chosen X-chromosome are silenced epigenetically early in development to allow expression of only one X-chromosome equivalent (lionization), thereby making female X-chromosome gene dosage largely equivalent to that of male subjects [8]. In the present study we aimed to diagnose the X-linked type of CGD in a group of Egyptian children by detection of CYBB gene expression using real time RT-PCR and to investigate if it correlates with the test of phagocytic lytic index as a cheaper diagnostic method for CGD.

Material and Methods

Subjects and sample collection:

This is case control study was conducted on 15 patients diagnosed with CGD, with a positive history of consanguinity, recruited from different university hospitals in Egypt. The laboratory work was conducted in Clinical Pathology Department, Immunology unit, Ain Shams University hospitals from September 2018 to July 2019. Protocol for this study was reviewed and approved by Ain Shams Faculty of Medicine, Ethical Committee and all the parents of the patients were provided by written informed Consent.

The study included the following groups:

- Group (I): Included 15 children (9 males, 6 females) provisionally diagnosed as CGD.

Inclusion criteria:

- Patients' age from 1 to 18 years old.
- Presented with recurrent infections
- Provisionally diagnosed chronic granulomatous disease (CGD) using the Dihydrorhodamine (DHR) test (retrieved from patient's files), stimulated by Phorbol Myristate Acetate (PMA), with stimulation index <30% [9].

Exclusion criteria:

- Known positive HIV serology or HIV nucleic acid testing.
- Active malignancy.
- Known myelodysplasia of the bone marrow or abnormal bone marrow cytogenetics.
- Group (II): Included 12 mothers to detect the genetic mutations in carriers, if any.
- Group (III): Included 14 normally apparent children (8 males, 6 females) as a control group.

Individuals included in the present study were subjected to complete history taking including consanguinity history and thorough clinical examination and laboratory workup which included; complete blood picture (for group I and group III) using three part cell counter (Mindray®, China), Phagocytic Lytic index (All study groups) and RT-PCR for CYBB gene expression (All study groups).

Blood sampling:

Four ml of venous blood were withdrawn under complete aseptic conditions into two sterile EDTA (1.2mg/ml) (Greiner ®) vacutainer tubes, for CBC and PCR; samples were transported in ice box in order to avoid any possible RNA degradation. RNA extraction was done and the extracts were stored at -80ºC until subsequent cDNA transformation. Two mL of venous blood were collected on a lithium heparin (Greiner ®) tube for Phagocytic Lytic index.

Phagocytic lytic index:

To assess the ability of neutrophils to phagocytose and lyse Candida albicans, it evaluates the ability of the neutrophils to generate ROS by which they kill the foreign invaders. Candida albicans were counted within 100 PMNs, we recorded both viable (deep purple-stained) and dead yeasts (blue-stained up to ghost), and the phagocytic and lytic indices were calculated from the following formulae **[10]:**

$$\text{Phagocytic index (PI)} = \frac{\text{Total number of phagocytosed candida / 100PMNs}}{100}$$

$$\text{Lytic index (LI)} = \frac{\text{Total number of dead candida / 100PMNs}}{100}$$

CYBB gene expression by reverse transcription PCR which included 3 steps:

Total RNA Isolation was done using a whole blood extraction kit (Qiagen® Germany, Cat. No 74104), followed by Two-Step RT-PCR using the QuantiTect Reverse Transcription (Qiagen®, Germany, Cat. No. 205311), according to the manufacturer instructions. Amplification and detection was done using QuantiTect SYBR Green PCR Kit (Qiagen® Germany Cat. No. 204143). The cycling condition included initial activation step at 95◦C for 5 minutes, then, denaturation follows at 95◦C for 5 seconds, thereafter, combined annealing/extension at 60ºC for 10 seconds for a total of 35 to 40 cycles. This was followed by analysis of the experiment and interpretation of results by viewing the amplification plots and setting the baseline and threshold values.

Interpretation was done using Relative Quantification (Calculation of fold change of gene expression using Delta CT, delta-delta CT and fold change formulae) as follows **[11]:**

- ΔCT = CT target gene – CT GAPDH (housekeeping gene).
- ΔΔ CT = ΔCT of patient or control sample – Mean of CT of control samples.
- Fold change = 2-ΔΔ CT.

Statistical analysis:

IBM SPSS statistics (V. 26.0, IBM Corp., USA, 2019) was used for data analysis. Date is expressed as M (median) and percentiles for quantitative non-parametric measures in addition to both number and percentage for categorized data. Mann Whitney Test was used to assess the statistical significance of the difference of a non-parametric variable between two study groups. Ranked Spearman correlation test was used to study the possible association between each two variables among each group for non-parametric data. The probability of error at 0.05 was considered significant, while at 0.01 and 0.001 were highly significant.

Results

This study is a case control study, conducted on fifteen patients (9 males, 6 females), provisionally diagnosed as CGD (Group I), with their age ranging from (3-15 years) with the median age (5 years) attending at different Egyptian university hospitals, their mothers underwent the same diagnostic tests, phagocytic index (PI), lytic index (LI), and RT-PCR (12 mothers - Group II), and 14 healthy children as a control group (Group III), and the descriptive statistics for the groups involved are shown below in Table (1).

Demographic and disease characteristics of the group (I) revealed that 100% of patients had positive history of consanguinity, 46% of cases showed onset of disease before 1 year of life and 54% after 1 year, 73% presented by pneumonia, 40% of cases presented by skin infections, 46% had partial or no response to treatment while 54% of cases experienced good response to treatment (Table 2).

Cases with a fold change of CYBB gene expression less than 0.34 (cut-off value calculated by the 25th percentile fold change of the control group), are considered having defective CYBB gene expression. At this cut-off value, three males (20% of the cases in group I and 33% of the males in group I) showed under-expression of the CYBB gene, while none of the females showed defective CYBB gene expression.

Case 1:

A 5 years old child who had a history of skin infections at the age of 2 years, and a history of recurrent abscesses that required repeated hospitalization with partial/no response to the frequently used antibiotics and hence prolonged hospitalization. His lab results showed that the fold change of CYBB gene expression was (0.1), the DHR test result was (2.36%), PI was (9.05%), LI was (3.25%), TLC was ($3.0 \times 10^3/\mu L$), ANC was ($0.8 \times 10^3/\mu L$) and the mother's fold change of CYBB gene expression was (12.1).

Case 2:

A 7 years old child was presented by pneumonia at the age of 2 years. He complained of repeated attacks of pneumonia that required hospitalization (3 times per year) with a good response to treatment. His lab results showed that the fold change of CYBB gene expression was (0.3) and the DHR test result was (6.2%), PI was (8.1%), LI (0.8%), TLC was ($3.2 \times 10^3/\mu L$), ANC was ($2.4 \times 10^3/\mu L$) and the fold change of CYBB gene expression of his mother was (3.86).

Case 3:

A 6 years old child was presented by pneumonia at the age of 6 months that required hospitalization (more than 5 times per year), at sampling he has fever (38ºC) and was on intravenous antibiotics. His lab results showed that the fold change of CYBB gene expression was (0.2) and the DHR test result was (2.65%), PI was (5.6%), LI was (0.42%), TLC was (4.7 x 10^3/uL), ANC was (4.0 x 10^3/uL) and the fold change of CYBB gene expression of his mother was (2.33).

Table (1): Descriptive statistics for various assessed parameters in the three included groups.

Parameter	Group I (n=15) Median IQR (25th-75th)	Group II (n=12) Median IQR (25^{th}-75^{th})	Group III (n=14) Median IQR (25^{th}-75^{th})
Age (Years)	5 (4-8)	–	6 (4-10.25)
Age of Disease Onset (Months-Years)	1 year (1 month – 3 years)		
TLC (x10^3/µL)	5.2 (3.9-8.7)	–	8.95 (6.02-12.47)
ANC (x10^3/µL)	2.4 (1.6-5)	–	4.8 (2.37-7.27)
PI (%)	6.8 (5.64-8.6)	9.55 (7.3-10.7)	8.97 (7.86-10.13)
LI (%)	0.8 (0.51-1.1)	1.24 (0.59-2.52)	1.3 (1.04-1.425)
DHR (%)	5.3 (2.22-10.0)		
Fold change of CYBB gene expression	1.7 (0.75-3.33)	2.18 (1.66-4.02)	1.13 (0.34-1.80)

Table (2): The Demographic and disease characteristics of Group (I).

Character	Group (I) (n=15)	
	No.	%
Males	9	60
Females	6	40
Consanguinity	15	100
Age of disease onset before 1 year	7	47
Age of disease onset after 1 year	8	53
Pneumonia	11	76
Skin Infection	6	40
No/Partial response to treatment	7	47
Good response to treatment	8	53

Discussion

Chronic granulomatous disease is an inherited disorder of the innate immune system characterized by a defective oxidative burst of phagocytes and subsequent impairment of their microbicidal activity. Mutations in one of the NADPH oxidase components affect gene expression or function of this system, leading to the phenotype of CGD. Children with CGD are often healthy at birth, but develop severe infections in infancy or early childhood. The most common form of CGD is genetically inherited in an X-linked manner, meaning it only affects boys. There are also AR forms of CGD that affect both sexes *[12]*.

In the present study we aimed to diagnose the X-linked type of CGD in a group of Egyptian children by detection of CYBB gene expression using real time RT-PCR and to investigate if it correlates with the test of phagocytic lytic index as a cheaper diagnostic method for CGD. This case-control study was conducted on 15 provisionally diagnosed CGD patients by the use of DHR test, with the stimulation index using PMA <30% *[9]*. They were recruited from different university hospitals in Egypt. The study also included twelve mothers of the studied patients to detect the genetic mutations in carriers, if any, and 14 apparently healthy children as a control group.

To the best of our knowledge this is the first study done to examine CYBB gene expression in terms of fold change from normal using real time RT-PCR technique.

Many laboratory technical methods have been used to aid in the diagnosis of CGD which include: (1) The evaluation of the phagocyte oxidative burst by NBT *[13]*, DHR *[14]* or the Candida albican phagocytosis *[10]*, (2) The molecular diagnosis either by using gene sequencing for mutation detection *[15,16]*, or by the conventional PCR technique *[12]*, (3) An immunological basis through the detection of the defective protein component of NADPH oxidase by the usage of the flow cytometry technique *[16,17]*.

CGD is usually diagnosed in childhood. The mean age of diagnosis for the X-linked recessive form is 3 years of age while the AR forms are diagnosed at 7.8 years of age *[18]*. A small number of CGD cases have been diagnosed in adulthood beyond the fourth decade *[19,20]*. The oldest case report

identified in the English language literature was of a male diagnosed at 69 years of age *[21]*. In our studied population, the median age at disease onset was one year old, some of them presented shortly after birth (1 month) with the maximum age of onset at 3 years old, which fell within a range of ages of onset reported by Kulkarni et al. *[16]* and El-Hawary et al. *[17]* who reported age of onset of disease at 8 months, and 19 months respectively. Wu et al. *[22]* studied 48 CGD patients and reported a mean onset of age as 0.29 years old, and 52% of their patients had an onset within the first month of life. Nevertheless, X-linked CGD carriers with mosaic pattern may present with milder symptoms in adulthood as stated by Kulkarni et al. *[16]*. Diagnosis may also be delayed because of newer potent antimicrobials that inadvertently treat many CGD-associated infections, postponing diagnosis until more severe infections indicate CGD as the underlying cause. X-linked CGD tends to have an earlier onset and be more severe than the most common AR form, p47phox deficiency *[20]*.

Genetic transmission in the majority of CGD males has been established as an X-linked recessive inheritance *[23]*. In family studies, the asymptomatic mothers can be identified as heterozygous carriers by intermediate in vitro leukocyte function, the fathers are normal, and some female relatives can be identified as carriers. The male to female ratio in this disease is found to be seven to one; which also suggests an X-linked transmission *[24]*. However, in the present study, nine out of 15 patients were males (60%) and 6 of them (40%) were females. These results were nearly similar to the corresponding ratios measured by El-Hawary et al. *[17]* who studied 28 CGD patients, of whom 15 were males (53%) and 13 were females (46.4%), and with those of Kulkarni et al. *[16]* who studied 90 CGD patients of whom 57 were males (63.3%) and 33 were females (36.6%).

In our study, we could reveal defective CYBB gene expression in 3 males out of the 15 patients in group I (20%) and out of the included 9 males in this group (33%). In contrast to our results, Florez et al. *[12]* could diagnose 7 patients out of 8 (87.5%) as X-CGD. The authors used conventional RT-PCR technique which they claimed it as a powerful tool to assess gene expression especially if the technique combines overlapping pair of primers to screen the full length of the gene. However, our results are in concordance with El-Hawary et al. *[17]*, who could diagnose 5 (17.9%) out of 28 patients as having X-CGD while the remaining 23 patients (82.1%) were diagnosed as AR-CGD. The authors assessed the NADPH protein expression by performing the intracellular staining with specific monoclonal antibodies on flow cytometry. They could conclude that in countries with limited resources and yet large number of CGD patients, the analysis of the defective proteins by flow cytometry is an optimum solution for confirming the diagnosis and is a step for targeted sequencing in families seeking prenatal diagnosis. Also, Kulkarni et al. *[16]* by using flow cytometry as a screening tool to identify the underlying affected gene, they could diagnose 27 patients (30%) out of 90 as X-CGD and the other 63 patients (70%) were diagnosed as AR-CGD. The difference between our results and others may be attributed to differences in the studied groups as regards to sample size and ethnicity and in the diagnostic characteristics of the analytical methods used, in terms of their sensitivity and specificity. The diagnostic characteristics of our technique using a cutoff of fold change of CYBB gene expression at 0.34 revealed that its sensitivity is 20%, its specificity is 86%, PPV is 60%, NPV is 50% and its accuracy is 52%. Our test showed low sensitivity, but high specificity and moderate accuracy. The low sensitivity is attributed to the inclusion of CGD cases with unknown genotyping.

In our study we could not detect CYBB gene under-expression in the six included CGD female patients which excludes CYBB gene abnormalities as a cause of CGD and which also necessitates the study of other subunit encoding genes of NADPH oxidase enzyme in these patients. Our results agreed with the finding that CGD in females *[25,26]*, and in some boys without demonstrable leukocyte defects in either parent *[27,28]*, has suggested another mode of inheritance. Genetic transmission in most females with CGD has been difficult to identify because neutrophils defects could not be demonstrated in either parent. In the few exceptions, leukocyte abnormalities were found in the mother but not the father. In one family, neutrophils from both the mother and a brother had intermediate responses for NBT reduction *[29]*, whereas in one other family *[30]*, only the mothers' neutrophils produced intermediate responses in NBT reduction, bactericidal activity, and cytochrome C reduction. In both families, the father had normal leukocyte function. The inability to detect the carrier state in families of females with CGD has suggested a non X-linked mode of transmission in these individuals. The use of insufficiently sensitive assays to measure neutrophil function may in part be responsible for the inability to detect neutrophil defects in mothers of either male or female CGD patients.

By the real-time RT-PCR technique used in our work we could not diagnose a female with X-CGD. However, X-CGD can affect females as verified by Kulkarni et al. *[16]*, they found a female patient who showed 8% positive neutrophils by NBT test and 10% positive neutrophils by DHR test, and showed heterozygous deletion of the CYBB gene detected by Sanger sequencing. They attributed their finding to be most probably due to extremely skewed X chromosome. Also, Eric et al. *[30]*, made a case study of a 16 years old female patient with X-CGD that underwent bi-directional sequencing of all the

13 exons of the CYBB gene and a flow cytometric DHR assay and showed extremely skewed X chromosome. These findings highlight the importance of gene sequencing in the diagnostic workflow of CGD patients.

In our study, the 12 mothers of the patients (Group II) showed normal CYBB gene expression. This finding may be explained by previous results of Repine et al. *[28]* who found that neutrophil bactericidal activity in asymptomatic mothers of males with the disease has shown a wide spectrum of activity ranging from normal to levels comparable with that of the patients themselves. The "Lyon hypothesis" could best explain how the occasional heterozygous females have or have not the clinical syndrome of CGD. By the Lyon hypothesis, there is random and permanent inactivation of one X-chromosome in somatic cells early in fetal development. The pattern of inactivation is inherited by all daughter cells *[31]*, should result in two distinct populations of neutrophils but no hybrid cells. Supporting this postulate, two populations of neutrophils have been identified in the peripheral blood of heterozygous females by NBT reduction *[23]* and by auto-radiographic studies of bacteria iodination *[32]*. Random inactivation of the X-chromosome would be expected to lead to an even distribution of normal and defective cells in heterozygous individuals. However, by chance, the distribution could vary, resulting in a few individuals with preferential inactivation of the defective X-chromosome and phenotypically near normal neutrophil function. This mechanism could explain the findings of normal neutrophil function in some mothers of either male or female patients. By the same process of random X-chromosome inactivation, a few individuals with preferential inactivation of the normal X-chromosome would be expected to have markedly abnormal neutrophils in which defective respiratory burst activity would be associated with decreased bactericidal activity and clinical disease. There is evidence from other X-linked diseases, i.e., glucose-6-phosphate dehydrogenase deficiency and hemophilia for non-random inactivation of the X-chromosome and clinical disease in heterozygous females *[33]*. Thus, females heterozygous for CGD may have near normal, intermediate, or severely abnormal neutrophil function. Depending upon the degree of their neutrophil dysfunction, they may be phenotypically normal individuals, or express mild to severe forms of the clinical syndrome.

All individuals carry at least 4-5 abnormal genes that are present in one copy. Parents who are close relatives (consanguineous) have a higher chance than unrelated parents to both carry the same abnormal gene, which increases the risk to have children with a recessive genetic disorder due to two copies of the same abnormal gene *[6]*. In the present study 100% of patients experienced positive family history of consanguinity. This finding came in co-ordinance with El-Hawary et al. *[17]* who found that (85.7%) of their studied patients had positive family history of consanguinity. In contrast,Kulkarni et al. *[16]* reported history of consanguinity in only (32%) of their patients.This difference may be attributed to the difference in sample size, different ethnicity and cultural habits.

In the present study, the most frequent presenting symptom in CGD patients was pneumonia (73%) while (40%) of them presented with skin infections. These findings came in concordance with Wu et al. *[22]* who found that the most prevalent infectious sites in their patients were the lungs (77%) followed by GIT (54%), lymph nodes (50%), and skin (46%). Yu et al. *[34]* stated that pneumonia is diagnosed in 70-80% of patients making it the most common infection overall while abscesses are another common initial presentation of CGD and can occur at any site. The authors recommend that children who present with fever and abscess, especially of the liver, perirectal, or perianal areas, should be evaluated for the disease.

In our study, about 46% of the patients experienced partial response to treatment with more frequent infectious attacks, meanwhile 54% of them showed good response with less frequent and less severe attacks. This may depend on the remaining ability of the neutrophils to generate ROS. A study published by Noack et al. *[35]*, involved 50 CGD patients, the authors found that 39 (78%) of them experienced more frequent attacks with partial response to the given antibiotics, while 11 of them (22%) showed better response with less frequent attacks. Kulkarni et al. *[16]* studied 90 CGD patients and they found that by history taking (33%) experienced partial response to the given treatment while (67%) experienced good response with a noticed increased prevalence of the partial response among the X-linked genotype.

In our work we could not find statistical significant difference in both TLC (median 5.2 $x10^3$/uL) and ANC (2.4 x 10^3/uL) in group I (patients group) when compared to group III (control group) (8.95 x 10^3/uL and 4.8 x 10^3/uL respectively), although both parameters are lower in the former group. We assumed that this result means CGD patient is not a must to be leucopenic, and the disease is a disorder of function not number. Kulkarni et al. *[16]* also found that the TLC range of their 90 patients were normal with a median TLC of 3.8 x 10^3/uL, same for El-Hawary et al. *[17]* who noticed that leucopenia is not a hallmark finding in their CGD patients.

The present study clarifies the importance of utilizing RT-PCR in analyzing the defective gene expression implicated in CGD as a method for confirming the genotypic diagnosis. However, it does not detect the mosaic pattern in carriers as NBT and DHR tests.

The NBT and the DHR tests have been considered the main tests used for evaluation of neutrophil respiratory burst. In spite that the NBT is not costly, the test is time-consuming, labor-intensive, subjective, and requiring technical skills. It also depends on cell number, pH and temperature **[25].**

While it can be argued that the NBT test still has a place in resource poor environment, said by Golightly **[36]**, the DHR flow cytometry test has then widely replaced the NBT in the diagnosis of CGD. The test is extremely robust, can be performed quickly using small volumes of anti-coagulated blood and inexpensive. The interpretation of the DHR test results is less subjective, and the test is able to differentiate between X-linked CGD patients and carriers **[17].** Although the DHR test has become the standard method for diagnosis of CGD, the test has some limitations. Important criticism for this method in routine diagnostics is its low sensitivity to in-vitro artifacts and neutropenia beside the fact that the neutrophils may be unevenly damaged due to storage, handling, or mailing **[37]**. The relatively short lifespan of neutrophils implicates that samples must arrive in the laboratory on the same day of collection which could certainly comprise a challenge for laboratories acting as tertiary referral centers **[38]**. The rarity of CGD has most likely influenced or precluded many clinical cytometry laboratories from thinking about or attempting to perform and bring the DHR test **[36].**

In our study we evaluated phagocyte oxidative burst by the use of slideneutrophil phagocytosis of Candida albicans and calculating both phagocytic and lytic indices **[10]**. This test is inexpensive, needs simple reagents that are usually available in every laboratory making it suitable to perform in less equipped laboratories. However, it needs well trained personnel as it is very subjective to interpret, labor-intensive test and should be performed on fresh blood samples and reagents.

The median PI of the cases in our work was found to be statistically significantly decreased (6.8%) when compared with that of the control group (8.9%) (p=0.005). This finding is contradicting with the fact that abnormal phagocyte function in CGD is associated with decreased bactericidal activity but ingestion of serum-opsonized organisms is reported to be normal in these patients. Gaither et al. **[39]** found that the PI is a sensitive test with a mean value for the cases of 2.13% and of 1.48% for the control group with a significant statistical comparison (p=0.05). Also, Hasui et al. **[40]** found that the ingestion of fluorescent bacteria by PMNs of CGD patients was significantly increased, that is about 1.5 times that of normal controls. This contradiction may be explained by the subjectivity of our technique or the cases included in this work may have other causes that affect phagocytic activity of neutrophils.

As regards the LI in our work, the median of the cases was 0.8% and that of the control group was 1.3% with a significant statistical decrease (p=0.026). These results agreed with many previous works that demonstrated defective killing of micro-organisms by CGD neutrophils(41-43). Our results did not show significant statistical correlations between fold change of CYBB gene expression and either of DHR%, PI% and LI% in the group of CGD patients (data are not shown in tables).

The major limitations of our study are: (1) The small sample size which is mainly influenced by the rarity and difficulty to find cases in a limited timeframe of the study, (2) Expensive reagents, (3) No available published researches that examined NADPH gene expression by the technique used in our study, so we have to compare the results of our technique by others' methodologies.

Conclusion:

By using real time RT-PCR technique we could establish the diagnosis of 3 out of 15 CGD cases as X-linked form, derives from defects in the CYBB gene, which encodes gp91phox of the oxidase, without the need to use complex and expensive methodologies such as northern blot, slot blot, or genomic DNA sequencing. Despite the low number of patients-included in this study to draw definite conclusions, this molecular analysis provides an insight into the breadth and relative distribution of genetic abnormalities responsible for the disease. Overall, we have demonstrated that RT-PCR, a simple and a relatively low cost methodology in comparison to other complex molecular diagnostic methods, may be a suitable tool for diagnosing CGD in laboratories in developing countries. It is very important to determine the definitive molecular genetic defect in order to provide the appropriate genetic counseling and prognosis to kindred with CGD. In addition, molecular genetic studies of the human NADPH oxidase system will advance the knowledge about this crucial and ancient defense mechanism and is a step towards national registry for primary immunodeficiency diseases.

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الكشف عن الظهور الجينى لجين (CYBB) على كروموسوم (X) كأداة لتشخيص داء الورم الحبيبى المزمن فى الاطفال المصريين

أمراض القصور المناعى الأوليه تنتج فى معظمها من عيوب جينية وتتميز بالاضطرابات فى واحدة أو أكثر من مكونات الجهاز المناعى، وعلى الرغم من التطور المعاصر فى الأدوات التشخيصية وسبل العلاج والبحث، يمر الجزء الأعظم من هذه الاضطرابات دون تشخيص خاصة فى البلدان الأقل تطورا.

داء الورم الحبيبى المزمن هو اضطراب فطرى، موروث، غير متجانس، يجعل المرضى عرضة للعدوى البكتيرية القيحية المتكررة أو الفطرية والالتهابات المفرطة مع تكوّن الورم الحبيبى فى النهاية والموت المبكر وتتسبب فى هذة الاضطرابات عيوب جينية فى انزيم NADPH oxidase تؤدى للخلل فى وظيفة واحدة أو أكثر من مكوناته ما يؤدى لنقص توليد فصائل الأكسجين النشطة التى تساهم فى قتل البكتريا.

وتبين أن الذكور هم الفئة الأكثر اصابة بهذا الداء وأثبتت الدراسات أن النوع المصاحب للكروموسوم اكس هو النوع الأكثر انتشارا بنسبة تبلغ سبعون بالمئة من الحالات، الانواع الأخرى المصاحبة للكروموسومات الجسدية تمثل نسبة أقل من ثلاثون بالمئة الا فى المناطق التى ينتشر فيها زواج الأقارب تزداد فيها نسبة باقى الأنواع التى تنتج نتيجة طفرة جينية فى الكروموسومات الجسدية ومصر هى احدى هذة البلدان.

ومن التحاليل المعملية المستخدمة للتشخيص هو تحليل صبغة التيترازوليوم الذى يعتمد على ترسيب الفورمازان داخل خلايا النيتروفيل عقب استثارتها وكلما قل ترسيب الصبغة زادت احتمالية كون المريض مصابا بالمرض و أيضا تحليل الهيدرو-رودامين الذى يعتمد على تغير لون صبغة الرودامين حال استثارة الخلايا وكلا الاختبارين يعتمد على تولد فصائل الأكسجين النشطة لتغيير اللون.

وتحليل صبغة الرودامين عن طريق استخدام تقنية التدفق الخلوى قادر على اكتشاف الأمهات الحاملة للمرض عن طريق وجود نوعين من خلايا النيتروفيل ذات انبعاث فلورسنتى مختلف على عكس الحالات المصابة التى تعجز خلاياها عن تغيير لون الصبغ وخلق الانبعاث الفلورسنتى.

ولكن ما يحد من استخدام التحاليل السابقة هو عدم تخصصية كليهما فى التشخيص لوجود العديد الانزيمات التى تنتج نفس الفصائل النشطة وتعطى نفس النتائج ما دفعنا فى دراستنا لدراسة المستوى الجينى فى التشخيص.

وأقيمت الدراسة على خمسة عشر مريضا أقل من ثمانية عشر عاماً وأمهاتهم ومجموعه أخرى مكونه من أربعة عشرة فرد كمجموعة تحكم، وخضعوا جميعها للكشف عن الظهور الجينز

وكان الهدف من الدراسة هو تقييم استخدام تقنية النسخ العكسى بواسطة تفاعل البلمرة المتسلسل كأداة تشخيصية لهذا المرض وثبت لدينا أنها ذات فعالية فى التشخيص عوضاً عن استخدام تقنية الهيدرورودامين.

خضع المرضى للاختبارات المعملية المذكورة قيد الدراسة وهى صورة الدم ومعدل الابتلاع و التحلل و تقنية انزيم النسخ العكسى، وتمت المقارنة بينهم احصائياً، وتبين عدم وجود دلالة احصائية بين المتغيرات كالسن وسن المريض عند بداية ظهور المرض، ومعدل الابتلاع ومعدل التحلل، وتم جمع نتائج الدراسة وتحليلها كما يلى:

تم تشخيص ثلاثة حالات من مجموع تسعة ذكور واجمالى خمسة عشر حالة، وهى تتفق مع النسب العالمية كون هذا الجين هو الأكثر تأثرا بالطفرات بين باقى الجينات التى تنسخ باقى مكونات الانزيم، وينصح الباحثون بامكانية استخدام وسائل أكثر دقة للتشخيص الدقيق لنوع الطفرات فى دراسات قادمة.

وبخصوص الأمهات (حوامل المرض) تبين عدم وجود أية نقص فى معدل التعبير الجينى لديهم و لكن هذا لا ينفى كونها حاملة للمرض باحدى الكروموسومين اكس ما ينتج عنه نقل الطفرة الجينية لابنائها الذكور فقط.

ولم نشخص من خلال دراستنا أى من الاطفال الاناث كونهن مصابات بخلل جينى فى الجين CYBB المتواجد على الكروموسوم اكس وهو لا ينافى احتمالية اصابة الاناث بخلل بهذا الجين عن طريق وجود حالة عدم تنشيط احدى الكروموسومين اكس وأثبتت عدة دراسات سابقة تلك الاحتمالية وأكدت احتمالية اصابة الاناث بهذا الخلل.

وبخصوص معدل الابتلاع والتحلل كانت نتائج المرضى أقل من نتائج مجموعة التحكم (الاطفال الأصحاء) وهو ما يناسب طبيعة المرض كونه خلل أكثر فى قتل البكتريا المبتلعة.

ايجازا، ينصح الباحثون باستخدام تقنية النسخ العكسى كونها تقنية دقيقة وسهلة الاستخدام فى المعامل ويوصى الباحثون باستخدام تقنية التسلسل الجينى مستقبلا كونها قادرة على تحديد الطفرة الجينية والخلل الجينى.