Research Article

Role of Real-Time polymerase chain reaction in diagnosis of Congenital adrenal hyperplasia

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Abstract
Background and objectives: The study is designed to detect of the copy number for Cyp21A2 gene in congenital adrenal hyperplasia (CAH) Egyptian children by real time polymerase chain reaction. **Subjects and methods:** The study was carried out on 188 subjects diagnosed clinically and biochemically as unrelated Egyptian CAH patients, registered as follow up cases of the Diabetes, Endocrine, Metabolic Pediatric Unit clinic (DEMPU), Cairo university hospitals. Each individual was subjected to history taking, clinical examination, laboratory investigations in the form of routine investigations; Na+, K+ and 17-OH Progesterone, and special investigation in the form of quantification of the CYP21A2 gene copy number using Real-Time PCR (RT-qPCR). **Results:** shows that out of 153 patients with low Na+ levels, 51 patients (33.3%) had (0 or 1) copy of the gene and 102 patients (66.7%) had ≥ 2 copies. This difference was statistically non significant (p=0.087). While, out of 145 ptients with high K+ levels, 50 patients (34.5%) had (0 or 1) copy of the gene and 95 patients (65.5%) had ≥ 2 copies. This difference was statistically significant (p=0.033). On the other hand, out of 174 patients with high 17-OH progesterone levels, 52 patients (29.9%) had (0 or 1) copy of the gene and 122 patients (70.1%) had ≥ 2 copies. This difference was statistically non significant (p=0.392).Also, shows that regarding 8bp deletion mutation diagnosis with differentiation between wild and mutant genotype, both techniques were concordant in 168 patients (89.4%). Cohen's kappa was 0.745, indicating substantial agreement between both methods in differentiating between wild genotype of 8bp deletion mutation and its mutant genotype. Compared to RT-qPCR, the sensitivity of Strip hybridization for diagnosing 8bp deletion mutation diagnosis with differentiation between wild and mutant genotype, was 87.7% and specificity was 90%. Overall accuracy was 89.4 %. **Conclusion**: RT-qPCR is easy to use in molecular diagnosis laboratory and together with the CYP21A2 gene sequencing might be a definitive way to detect almost all common as well as rare 21OHD alleles.
Key words: CYP21A2, RT-qPCR, CAH.

Indroduction
Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21OHD) is an autosomal recessive disorder caused by an inborn error of steroid metabolism and accounts for 90–95% of all CAH cases [(1)].

The genetics of CYP21A2 are unusual and complicated. Random deletions and de novo mutations almost don't occur, instead, gene conversions, accounts for about 85% of all mutant CYP21A2 alleles. In these gene conversions, all or part of the CYP21A2 gene is replaced by, or converted to, the sequence of the corresponding CYP21A1P [(2)].

Molecular diagnosis of the disease can be performed with direct methods for identifying mutations; several protocols in which selective amplification of the active gene is performed before screening for different pathological mutations have been proposed [(3)]. In a group of Egyptian CAH patients rapid methods for direct detection of mutations were done [(4)].

Several different methods that can identify CYP21A2 mutations, such as the amplification-created restriction site approach, multiplex minisequencing and direct gene sequencing, have been applied to CAH genotyping [(2)]. Chromosomal rearrangements have been investigated by Southern blotting quantitative real-time PCR and multiplex ligation-dependent probe amplification (MLPA) [(5)].

The aim of this work is to detect of the copy number for CYP21A2 gene in congenital adrenal hyperplasia (CAH) Egyptian children by real time polymerase chain reaction.

Subjects And Methods

The study was conducted on a total number of 188 Egyptian children subjected to strip hybridization assay as a part of Science and Technological Development Fund project (STDF). The patients' age at time of sample collection varied from 14 days to 19 years. The patients' age at their first presentation varied from day of birth to 7 years. Consanguinity was reported in 132 families. The patients were subjected to Full history taking, Clinical examination for CAH symptoms & signs including assessment of the degree of genital ambiguity by Prader staging according to (Harris and Wayne, 2006). Strip hybridization assay for the common 11 mutations in CYP21A2 gene using CAH Strip Assay® (Viennalab Diagnostics, Austria) and All patients were subjected to copy number detection with gene dosage assessment by real time quantitative PCR (Rt-qPCR) [(5)].

Sampling protocol:

Venous blood was collected from each patient by sterile venipuncture under complete aseptic conditions at the time of routine laboratory investigations. Blood was collected in a sterile EDTA vaccutainer for genotyping techniques. DNA extraction was done from fresh whole blood samples using. Then, the extracted DNA was stored at -20ºC till amplification followed by reverse hybridization assay and CYP21A2 gene copy number detection with gene dosage assessment by real time PCR (Rt-qPCR) (Applied Biosystem, USA).

Laboratory investigations:

DNA extraction was done using QIAamp DNA blood Mini kit-Qiagen (Qiagen, Germany) according to the manufactural instructions Adapted from (QIAamp DNA Mini Kit Handbook).All DNA samples subjected to reverse hybridization assay performed through CAH reverse strip hybridization assay® (Viennalab Diagnostics, Austria).then CYP21A2 gene Copy number detection using real time PCR (Applied Biosystem, USA).

All analyses were performed with version 19 of Statistical Package of Social Science (SPSS).Qualitative data were expressed as proportions, while quantitative data were expressed as mean + standard deviation (SD). Qualitative data were analyzed by Chi square ($\chi 2$) test. Comparisons between groups for normally distributed quantitative data were performed by Student's t-test. Correlations between variables were obtained by Pearson's test. For all analyses, statistical significance was defined as p values less than 0.05.

Results

Demographic data for the studied group are shown in table (I). 70.2% of the studied children with 21-OHD were with positive consanguinity, 19.1% of the patients had similar cases among siblings while 15.4% of the patients had sibling death in their family and 8.5% reported both siblings affection and death. Serum sodium levels were normal in 18.6% of the patients and low levels were seen in 81.4%. Serum Potassium levels were normal in 22.3% of the patients and high levels were seen in 77.7%. Serum levels of 17-OH proges-terone were elevated in 93.6% of patients and normal levels were in 6.4%.Salt wasting clinical presentation was seen in 76.6% of the cases while simple virilizing clinical presentation was seen in 23.4% of enrolled subjects as shown in tables (II). the copy number results detected by Real-time PCR. Where, 116 patients (61.7%) had 2 copies of CYP21A2 gene, 17 patients

(9.0%) had 1 copy, 12 patients (6.4%) had 3 copies, 1 patient (0.5 %) had 4 copy and 42 patients (22.4 %) had gene deletion shown in table (III).

Regarding 8bp deletion mutation diagnosis with differentiation between wild and mutant genotype, both techniques were concordant in 168 patients (89.4%). Cohen's kappa was 0.745, indicating substantial agreement between both methods in differentiating between wild genotype of 8bp deletion mutation and its mutant genotype. Compared to RT-PCR, the sensitivity of Strip hybridization for diagnosing 8bp deletion mutation diagnosis with differentiation between wild and mutant genotype, was 87.7% and specificity was 90%. Overall accuracy was 89.4 % as shown in table (IV).

Table (I) Demographic Characteristics of the studied groups

Demographic data		Cases (n=188)
Age (years)*		6.2 ± 3.2
Gender!	**Female**	131 (69.7%)
	Male	57 (30.3%)

* Data presented as mean ± standard deviation for age at time of sample collection.
! Data presented as number (Percent)

Table (II): Frequency distribution of clinical and laboratory data

		Frequency (n1=188)	%
Consanguinity	**Negative**	56	29.8
	Positive	132	**70.2**
Similar cases in siblings (%)	**Absent**	107	**56.9**
	Affected	36	19.1
	Death	29	15.5
	Both (one affected & one dead)	16	8.5
Sodium (mmol/L) (Na^+)	**Normal** 2	35	18.6
	Low	153	**81.4**
Potassium (mmol/L) (K^+)	**Normal 3**	42	22.3
	High	146	**77.7**
17-OH Progesterone (ng/ml)	**Normal 4**	12	6.4
	High	176	**93.6**
Clinical presentation	**Salt wasting**	144	**76.6**
	Simple virilizing	44	23.4

1 n= number of subjects.
2 Reference range for sodium = 135-146 mmol/L**.**
3 Reference range for potassium in children =3.5- 5.1 mmol/L.
4 Normal 17-OH Progesterone level ≤ 10 ng/ml.

Table (III): Results of gene dosage analysis of CYP21A2 gene copy number detected by Real-time PCR

	Frequency	%
1 copy	17	9.0
2 copy	116	**61.7**
3 copy	12	6.4
4 copy	1	0.5
Gene deletion (0 copy)	42	22.4

Table (IV): McNemar test between Strip hybridization test and RT-qPCR in diagnosis of 8bp deletion mutation (Wild, Mutant)

	RT-qPCR		Total	Cohen's kappa	P.Value
Strip	**Wild**	**Mutant**			
Wild	118	7	125	0.745	<0.001*
Mutant	13	50	63		
Total	131	57	188		

Discussion

Congenital adrenal hyperplasia (CAH) is a common autosomal recessive disorder that is frequently caused by 21-hydroxylase deficiency (21-OHD) (90% of cases). Impaired 21-OH enzyme activity lead to a deficiency in adrenal cortisol and aldosterone production, increase in androgen secretion and renal salt loss [(6)] .

Patients with CAH can be diagnosed on the basis of biochemical assessment of hormonal metabolites of 17-OHP, the metabolite immediately preceding the 21 hydroxylation step in steroidogenesis. Also molecular analysis of CYP21A2 gene showed that 21OHD CAH is associated with distinct genotypes chacterized by varying enzyme activity [(7)]. However, direct assessment of the enzymatic activity of 21-OH is impossible because CYP21A2 gene is expressed principally in the adrenal cortex [(1)].

This study included method evaluation study conducted to confirm the results of Strip hybridization method for patients encountered in a group of Egyptian children diagnosed on clinical and hormonal basis as 21-OHD CAH from those attending diabetes, endocrine and metabolic Pediatric unit (DEMPU) , Children Hospital , Cairo University. Detection of CYP21A2 gene copy number was done using real-time polymerase chain reaction (RT-qPCR).

In the present study, serum sodium levels were normal in 18.6% of the patients, while low levels were seen in 81.4%. Serum potassium levels were normal in 22.3% of the patients, while high levels were seen in 77.7%. Impairment of 21 -OH enzyme activity in cases with CAH leads to deficiency of adrenal cortisol and aldosterone that causes inability to retain sodium and excrete potassium from the renal tubules[(8)].Similarly, Larissa et al., (2013) [(9)] stated that at time of diagnosis, all of the patients had hyponatremia and hyperkalemia. Also, Firdevs et al., (2009)[(10)] stated that the criteria used to diagnose a SW form were either a SW crisis in the newborn period or elevated plasma renin activity (PRA) levels, hyponatremia and hyperkalemia.

In the current study, serum levels of 17-OH progesterone were elevated in 176 cases (93.6%) and normal in 12 cases (6.4%). Similarly, Torres and co-workers, (2003)[(11)] reported that the SW form was characterized by extremely elevated concentrations of 17OHP, hyponatremia and hyperkalemia in the first days of months of life and this was agreed upon by New et al., (2014)[(12)] and Wedell et al., (2011) [(13)].

The current study showed that regarding 8bp deletion mutation diagnosis with differentiation between wild and mutant genotype, both techniques were concordant in 168 patients (89.4%). Cohen's kappa was 0.745, indicating substantial agreement between both methods in differentiating between wild genotype of 8bp deletion mutation and its mutant genotype. Compared to RT-qPCR, the sensitivity of Strip hybridization for diagnosing 8bp deletion mutation diagnosis with differentiation between wild and mutant genotype, was 87.7% and specificity was 90%. Overall accuracy was 89.4%

RT-qPCR method offers an accurate alternative to Southern blot and other methods described previously for 21OHD diagnosis and has the following advantages: (a) it requires only a small amount of DNA (50–100 ng); (b) it is neither time-consuming (2 h) nor laborious, and can analyze many samples simultaneously; and (c) it is effective in the detection of CYP21A2 gene duplication, which is one of its most important advantages [(14)].

Conclusion

RT-qPCR is easy to use in molecular diagnosis laboratory and together with the CYP21A2 gene sequencing might be a definitive way to detect almost all common as well as rare 21OHD alleles.

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