RESEARCH PAPER
INTERNATIONAL JOURNAL OF PURE MEDICAL RESEARCH

Clinical Science

KEYWORDS: CAH, CYP21A2, Strip hybridization,

CONGENITAL ADRENAL HYPERPLASIA DIAGNOSIS STILL A CHALLENGE

Volume-2, Issue-7, July - 2017

Ahmad A. Omran Clinical Pathology Department, Faculty Of Medicine, Minia University.

S. Sharaf!, M. Hafez Pediatric Medicine Department, Faculty Of Medicine, Cairo University.

Aliaa M. Higazi* Clinical pathology department, faculty of medicine, Minia University. *Corresponding Author mahmoud.znaty@yahoo.co

Zaki M. Zaki Clinical pathology department, faculty of medicine, Minia University.

Article History
Received: 02.04.2017
Accepted: 10.06.2017
Published: 10.07.2017

ABSTRACT:

Background: Congenital adrenal hyperplasia is one of the main pediatric referent, due to genital ambiguity and mortality of neonates as a result of dehydration and shock. The matter is exacerbated by high degree of consanguinity in our population. Newborn screening for CYP21A2 gene mutations can minimize delay in diagnosing, reducing morbidity and mortality from adrenal salt-wasting crises. Therefore, there is a need to establish a rapid, convenient prenatal screening method for diagnosis of CAH cases in Egypt.

Objective: The study is designed for reversed hybridization method for patients previously typed positive for 8bp deletion encountered in 188 Egyptian children diagnosed on clinical and hormonal basis as 21-OHD from those attending diabetes, endocrine and metabolic Pediatric unit (DEMPU), Children Hospital, Cairo University.

Conclusion: Reversed Strip hybridization assay is a reliable method for diagnosis of mutations in CAH patients and has many advantages being easier and can detect more than one mutation at a time.

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 (White and Speiser, 2000).

The CYP21A2 gene encodes the enzyme steroid 21-hydroxylase (21OH), which is essential for steroid synthesis in the adrenal cortex. Mutations in this gene are the main cause of CAH (Speiser et al, 2010).

The classical forms of CAH are the salt wasting (SW) and simple virilising (SV) phenotypes with adrenocorticotropic hormone-driven excess of androgens leading to ambiguous genitalia in female newborns. The SW phenotype has complete or partial loss of enzyme activity, leading to lack of cortisol and aldosterone, whereas the SV displays various degrees of reduced enzyme activity resulting in cortisol insufficiency (Wedell, 1998).

The non-classical (NC) CAH is milder and characterized by signs of hyperandrogenism postnatally and in adulthood, and associated with minor mficiency (Wedell, 1998).

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 (Concolino et al, 2010).

Several different methods that can identify CYP21A2 mutations, such as the amplification-created restriction site approach (Lee et al, 1996), multiplex minisequencing (Krone et al, 2002) and direct gene sequencing (Jeske et al,2009), have been applied to CAH genotyping. Chromosomal rearrangements have been investigated by Southern blotting (Concolino et al, 2009), quantitative real-time PCR (Parajes et al, 2007) and multiplex ligation-dependent probe amplification (MLPA) (Concolino et al, 2009). Direct sequencing in combination with MLPA offers the highest diagnostic information, however, being very time-consuming and costly, does not represent an attractive second-tier test for newborn screening programs at this stage (Malikova et al, 2012).

al., 1999; Krone et al., 2000). In a group of Egyptian CAH patients rapid methods for direct detection of mutations were done (Sahar et al., 2014)

The CAH Strip Assay provides a fast (7 h) and reliable protocol to identify common CYP21A2 mutations. Only small amounts of DNA are required, allowing analysis from dried blood spot punches (Németh et al., 2012) Several different methods that can identify CYP21A2 mutations, such as the amplification-created restriction site approach (Lee et al., 1996), multiplex minisequencing (Krone et al., 2002), and direct gene sequencing (Kösel et al.,

2005; Jeske et al.,2009), have been applied to CAH genotyping. Chromosomal rearrangements have been investigated by Southern blotting (Baumgartner-Parzer et al., 2003; Concolino et al., 2009), quantitative real-time PCR [Parajes et al., 2007; Olney et al., 2002) and multiplex ligation-dependent probe amplification (MLPA) (Concolino et al., 2009). Direct sequencing in combination with MLPA offers the highest diagnostic information, however, being very time-consuming and costly, does not represent an attractive second-tier test for newborn screening programs at this stage (Malikova et al., 201 l., 1999; Krone et al., 2000). In a group of Egyptian CAH patients rapid methods for direct detection of mutations were done (Sahar et al., 2014)

The CAH Strip Assay provides a fast (7 h) and reliable protocol to identify common CYP21A2 mutations. Only small amounts of DNA are required, allowing analysis from dried blood spot punches (Németh et al., 2012) Several different methods that can identify CYP21A2 mutations, such as the amplification-created restriction site approach (Lee et al., 1996), multiplex minisequencing (Krone et al., 2002), and direct gene sequencing (Kösel et al., 2005; Jeske et al.,2009), have been applied to CAH genotyping. Chromosomal rearrangements have been investigated by Southern blotting (Baumgartner-Parzer et al., 2003; Concolino et al., 2009), quantitative real-time PCR [Parajes et al., 2007; Olney et al., 2002) and multiplex ligation-dependent probe amplification (MLPA)

(Concolino et al., 2009). Direct sequencing in combination with MLPA offers the highest diagnostic information, however, being very time-consuming and costly, does not represent an attractive second-tier test for newborn screening programs at this stage (Malikova et al., 201

AIM OF THE WORK

The study was designed to confirm the results of reversed strip hybridization method for CYP21A2 in 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.

Subjects And Methods

This study was conducted on children clinically diagnosed as CAH with 21-OHD. They were selected from patients regularly attending the follow up at the Diabetic and Endocrine Metabolic Pediatric Unit, Children Hospital, Cairo University (DEMPU), Egypt.

This study was performed in Egyptian children were previously screened for 11 mutations by reversed strip hybridization assay and to confirm the gene deletions and hemizygosity in homozygous mutations detected by strip hybridization method.

2.1. Subjects

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.

2.1.1. Inclusion Criteria

1) Patients of both sexes with clinical diagnosis of CAH due to 21-OHD (salt wasting or simple virilising) were included in the current study.

2) Patients were laboratory diagnosed as having CAH by elevated 17-hydroxyprogesterone (Nimkarn and New, 2010).

2.1.2. Exclusion Criteria

Patients who were diagnosed as CAH not due to 21-OHD were excluded.

2.1.3. The patients were subjected to the following:

1- Full history taking including family history.

2- Clinical examination for CAH symptoms & signs including assessment of the degree of genital ambiguity by Prader staging according to (Harris and Wayne, 2006).

3- Routine investigations that used for diagnosis of CAH cases including steroid hormonal specific profile (17α-OHP, DHEA, Δ 4-androstenedione, testosterone and plasma renin levels) detected by immunoassay analyzer® (Immulite 1000, USA) along with karyotyping.

4- Strip hybridization assay for the common 11 mutations in CYP21A2 gene using CAH Strip Assay® (Viennalab Diagnostics, Austria).

2.2. Methods

2.2.1. Samples Collection

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. Then, the extracted DNA was stored at -20 °C till amplification followed by reverse hybridization assay (Applied Biosystem, USA).

2.2.2. DNA Extraction

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).

2.2.2.1. Principle

The extraction of DNA from whole blood encompassed the lysis of proteins, nucleases and contaminants by proteinase K enzyme with the lysis buffer. DNA purification was carried out using QIAamp Spin Columns. The lysate buffering conditions allowed optimal binding of the DNA to the QIAamp membrane as soon as the sample was loaded into the Spin Column. DNA was adsorbed onto the QIAamp silica-gel membrane during a brief centrifugation. Salt and pH conditions in the lysate ensured that proteins and other contaminants, which could inhibit PCR, were not retained on the membrane. DNA bound to the membrane was washed by two different wash buffers in two centrifugation steps to improve the purity of the DNA. Purified DNA was eluted from the QIAamp spin column in a concentrated form in elution buffer (Fode et al, 2011).

2.2.2.2. Blood Spin Protocol

1. Whole blood 200 µL were added to 20 µL QIAGEN protease.

2. Buffer AL 200 µL were added to the sample and all mixed by pulse-vortexing for 15 seconds.

3. The microcentrifuge tube was incubated at 56 °C for 10 minutes.

4. The microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.

5. Ethanol (96-100%) 200 µL were added to the sample and mixed again by pulse-vortexing for 15 seconds. After mixing, the microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.

6. The mixture from step 5 was carefully applied to the QIAamp Spin Column (in a 2 mL collection tube) without wetting the rim. The cap was closed and then the spin column was centrifuged at 8000 rpm for 3 minutes. The QIAamp Spin Column was placed in a clean 2 mL collection tube and the tube containing the filtrate was discarded.

7. The QIAamp Spin Column was carefully opened and 500 µL Buffer AW1 was added without wetting the rim. The cap was closed and the Spin Column was centrifuged at 8000 rpm for 3 minutes. The QIAamp Spin Column was placed in a clean 2 mL collection tube and the tube containing the filtrate was discarded.

8. The QIAamp Spin Column was carefully opened and 500 µL Buffer AW2 was added without wetting the rim. The cap was closed and centrifuged at full speed 14.000 rpm for 5 minutes.

9. The QIAamp Spin Column was placed in a new 2 mL collection tube and the collection tube with the filtrate was discarded. Centrifugation at full speed 14.000 rpm for 3 minutes was done.

10. The QIAamp Spin Column was placed in a clean 1.5 mL microcentrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp Spin Column was carefully opened and 200 µL Buffer AE was added. Incubation for 5 minutes at room temperature (to increase DNA yield) and then centrifugation at 8000 rpm for 3 minutes were done.

11. DNA eluted in buffer AE was stored at – 20 °C till amplification.

2.2.2.3. Quantification of DNA

DNA concentration of the sample was then measured on Qubit® 2.0 Fluorometer according to (Acar et al, 2009). This device uses fluorescent dyes to determine the concentration of nucleic acids (DNA and RNA) and proteins in a sample with a dye specific for each. The UV-absorbance method uses a spectrophotometer to measure

the natural absorbance of light at 260 nm (for DNA) (McKnight et al, 2006).

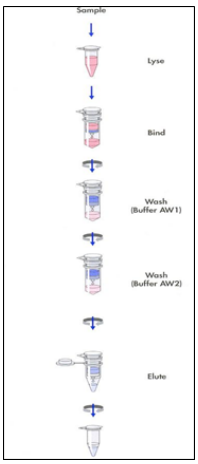


Figure 22: QIAamp Spin procedure (Adapted from QIAamp DNA Mini Kit Handbook)

Steps of DNA quantification by Qubit® 2.0 Fluorometer:

1. Preparation of working buffer:
Qubit dsDNA HS Buffer: [Number of samples+3] x199µl = _____
Qubit Reagent (fluorophore): [Number of samples+3] x1µl = _____ (The extra 3 samples allow for 2 standards and for pipetting errors)

2. The working buffer was mixed by vortexing.

3. Qubit Assay tubes were labeled with sample ID

4. For each sample, 2µl of PCR product were added to 198µl of working buffer to the appropriate tube

5. For each of the two standards, 10µl of standard were added to 190µl of working buffer to the appropriate tube

6. Each sample was vortexed for 2-3 seconds to mix then incubated for 2 minutes at room temperature.

7. On the Qubit fluorometer, DNA was hit, then dsDNA High Sensitivity, then YES.

8. When directed, standard 1was inserted, the lid was closed, and Read was hit.

9. Each sample was read by inserting the tube into the fluorometer, closing the lid, and hitting Read Next Sample.

10. DNA concentration is auto-calculated and appeared on the screen as ng/µl

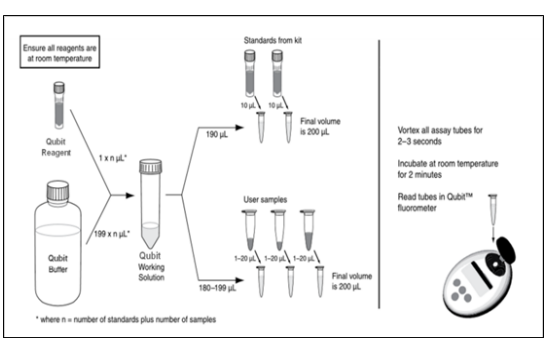


Figure 23: Protocol of DNA quantification by Qubit® 2.0 Fluorometer Adapted from (QIAamp DNA Mini Kit Handbook)

2.2.3. Reverse Hybridization Assay

All DNA samples subjected to reverse hybridization assay which is an assay for the identification of mutations associated with CAH based on polymerase chain reaction (PCR) and reverse-hybridization (Németh et al, 2012). Reverse hybridization assay was performed through CAH reverse strip hybridization assay® (Viennalab Diagnostics, Austria).

This procedure includes three steps: (1) DNA isolation, (2) PCR amplification using biotinylated primers, (3) hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences are detected using streptavidin-alkaline phosphatase and color substrates (Németh et al, 2012). The assay covers 11 common mutations in the CYP21A2 gene: p. P30L, I2 splice (I2 G), Del 8 bp E3 (G110del8nt), p.I172N, Cluster E6 (p.I236N, p.V237E, p.M239K), p.V281L, p.L307 frameshift (F306+T), p.Q318X, p.R356W, p.P453S, p.R483 (Németh et al, 2012).

When the strip showed failure of hybridization of mutant and wild probes the results interpretation according to manufacturers instructions are :-

- Failure of the PCR reaction OR
- Whole gene deletion

When the strip showed homozygous mutation, its interpretation is according to the copy number:

- Hemizygous if one copy of the gene
- Homozygous if two copies of the gene.

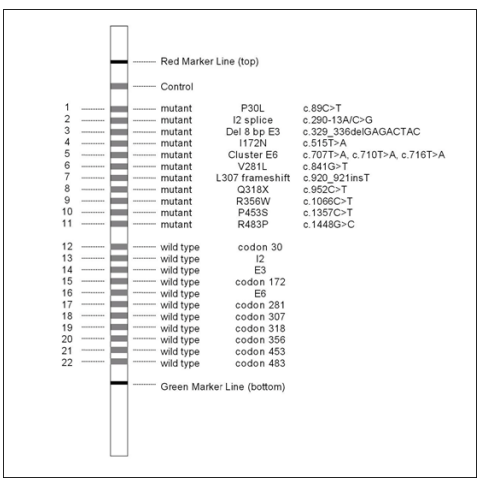


Figure 24: CAH reverse strip hybridization assay® Adapted from (Viennalab Diagnostics, Austria)

Table (2): PCR reaction mixture for amplification of CYP21A2 gene

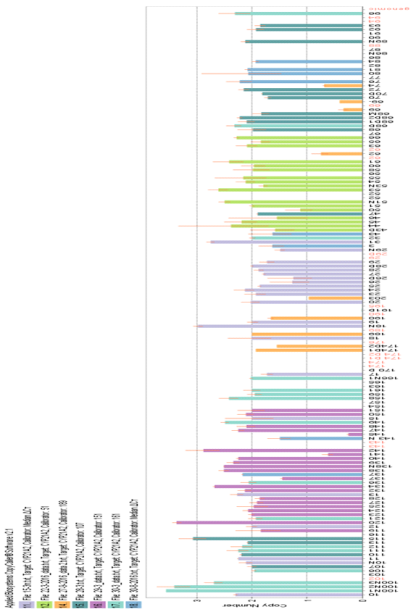
	Stock Solutions	Volume
Master mix*	(i-Taq)(2x) PCR solution	10 L
Distilled water	Nuclease-free water	2 L
Primers (FAM) (diluted 1:10)	Primer F Primer R	1 L 1 L
Probe (FAM)	(diluted 1:33)	1 L
Primers (VIC) (diluted 1:10)	Primer F Primer R	1 L 1 L
Probe (VIC)	(diluted 1:33)	1 L
DNA	Genomic extracted DNA (20ng/µl)	2 L
Total volume		20 L

* Master mix used is the same for all the study reactions, (i-Taq) (2x) PCR solution (Applied Biosystems, USA).

STATISTICAL METHODS:

Statistical analysis was done using IBM© SPSS© Statistics version 22 (IBM© Corp., Armonk, NY, USA). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non parametric t-test).

Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA) then post-Hoc was used for pair-wise comparison based on Kruskal-Wallis distribution. Spearman-rho method was used to test correlation between numerical variables. All tests were two-tailed. A p-value < 0.05 was considered significant.



The study was conducted on a total number of 100 Egyptian children with CAH 21-OHD (40 males and 60 females) from those regularly attending the follow up at the Diabetic and Endocrine Metabolic clinic, Children hospital, Cairo University (DEMPU).

Table (4): 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 (4) shows that 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 progesterone 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.

Table (7): Frequency distribution of 8bp deletion mutation genotyping in CYP21A2 gene detected by strip hybridization test

	Frequency	%
Gene deletion	21	11.2
Wild pattern (No mutation)	126	67.0
Heterogenous pattern (mutation in one allele)	19	10.1
Homogenous pattern (mutation in two alleles)	22	11.7

Table (7) shows the frequency of 8bp deletion mutation that was diagnosed by strip hybridization test. The frequency of absent gene (or failed reaction) were 11.2%, frequency of wild pattern (No deletion) were 67.0%. Additionally, the frequency of mutation 41 patient (21.8%); heterogenous pattern (mutation in one allele) was 10.1% and the frequency of homogenous pattern (mutation in two alleles) was 11.7%.

Table (9): Association between karyotype (definitive sex) and clinical type

		Clinical type		Total	p-value
		Salt wasting	Simple virilizing		
Karyotype	♀	93 (71%)	38 (29%)	131 (100%)	0.239*
	♂	51 (89.5%)	6 (10.5%)	57 (100%)	
Total		144 (76.6%)	44 (23.4%)	188 (100%)	

*Significant level at P value < 0.05

Table (9) shows that out of the 131 female patients , 93 (71%) were salt wasting and 38 (29%) were simple virilizing. On the other hand, out of 57 male patients, 51 (89.5%) were salt wasting and 6 (10.5%) were simple virilizing . This difference was statistically non-significant (p=0.239).

Table (10): Association between 8bp deletion mutation and clinical type

8bp deletion mutation by strip hybridization		Clinical type		Total	p-value
		Salt wasting	Simple virilizing		
Gene deletion		20 (13.9%)	1 (2.3%)	21 (11.2%)	0.004*
Normal (wild)	94 (65.3%)	32 (72.7%)	126 (67 %)		

Mutant	Heterozygous	10 (6.9%)	9 (20.5%)	19 (10.1%)	
	Homozygous	20 (13.9%)	2 (4.5%)	22 (11.7%)	
Total		144 (100%)	44 (100%)	188 (100%)	

*Significant level at P value < 0.05

Table (10) shows that out of 126 patients who were negative for 8bp deletion mutation, 94 patients (65.3%) were salt wasting and 32 patients (72.7%) were simple virilizing. While, out of 41 patients who were positive for 8bp deletion, 30 (20.8%) were salt wasting and 11 (25%) were simple virilizing. On the other hand, from 21 patients who had no gene, 20 (13.9%) were salt wasting and 1 (2.3%) were simple virilizing. This difference was statistically significant (p=0.004).

Table (11): Association between 8bp deletion mutation and Laboratory data (17-OH Progesterone)

8bp deletion mutation by strip hybridization		Laboratory data (17-OH Progesterone) (ng/ml)		Total	p-value
		Normal (≤ 10)	High (> 10)		
Gene deletion		0 (0%)	21 (11.9%)	21 (11.2%)	0.569*
Normal		9 (75.0%)	9 (75.0%)	126 (67 %)	
Mutant	Heterozygous	2 (16.7%)	17 (9.7%)	19 (10.1%)	
	Homozygous	1 (8.3%)	21 (11.9%)	22 (11.7%)	
Total		12 (100%)	176 (100%)	188 (100%)	

*Significant level at P value < 0.05

Table (11) shows that out of 126 patients who were negative for 8bp deletion mutation, 9 patients (75.0%) had normal 17-OH Progesterone levels and 117 patients (66.5%) had high 17-OH Progesterone levels. While, out of 41 patients who were positive for 8bp deletion mutation, 3 (25.0%) had normal 17-OH Progesterone levels and 38 patients (21.6%) had high 17-OH Progesterone levels. On the other hand, all patients who had no gene, had high 17-OH Progesterone levels. This difference was statistically non significant (p=0.569).

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 (Zarger et al, 2016).

The variable clinical phenotypes as classic form, consist of salt wasting (SW) and simple virilizing (SV) type, and non classic form (late onset) (LO) (forms, depend on the reduced enzymatic activity due to different combination of gene mutations which may lead to mortality and morbidity (Rabbani et al, 2011). In the SW form there is complete lack of the 21-OH enzyme activity, in the SV form there is partial impairment of the 21-OH enzyme , while in the LO form there is mild impairment of the 21-OH enzyme (Bas et al, 2009).

Girls affected with CAH showed a wide spectrum of virilization from mild to severely virilized external genitalia (0–5 Prader score) and could be raised up as males by mistake. The most common cause of ambiguous genitalia in the newborn is CAH, 1 per 15,000 live births (Abou El Ella et al, 2016). In addition, insufficiency of aldosterone seen typically in the first 2 weeks of life, leads to signs of SW form with hyponatremia, hyperkalemia, diarrhea, vomiting, shock and death (Razzaghi-Azar et al, 2002).

On the other hand, affected boys revealed excess androgens and depending on the severity of the enzyme defect, displayed hyponatremia and hyperkalemia, which follow diarrhea, vomiting, gastroesophageal refluxes, and failure to thrive. Hyponatremia and hyperkalemia were observed in those who lacked aldosterone. Patients with severe deficiency of cortisol had increased ACTH with hyperpigmentation and virilization (Rabbani et al, 2012).

In non-classic CAH, patients are asymptomatic at birth, and mostly manifest in later on with precocious pseudopuberty or a clinical picture resembling polycystic ovary syndrome (PCOS) comprising hirsutism, oligomenorrhea and anovulation (Krone, 2013).

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 characterized by varying enzyme activity (Choi et al, 2016). However, direct assessment of the enzymatic activity of 21-OH is impossible because CYP21A2 gene is expressed principally in the adrenal cortex (White and Speiser, 2000).

High concentration of serum 17-OHP is observed in classic 21-OH deficiency ,however, false-positive results can be generated in pre-term or low birth- weight newborns or in association with unrelated neonatal diseases. In such cases, follow up of 17-OHP levels is needed, which increases the cost of screening programs and causes anxiety for parents (Silveira et al, 2009).

The CYP21A2 gene has been mapped on chromosome 6p21.3. This gene consists of ten exons and is 98% similar to its pseudogene, CYP21A1P. This similarity leads to intergenic recombination events, accounting for 75% of the microconversion-derived mutations. About 20%–25% of the recombinations are due to unequal crossing over, causing large gene conversions, duplications, and deletions (Rabbani et al, 2012).

According to Human Gene Mutation Database (HGMD) there are more than 100 mutations found. The following common mutations have been described in many populations accounting for three forth of the mutations: g.659A/C>G (I2G), g.707_714delGAGACTAC (p.G110_Y112), g.1004>A (p.I172N), g.89C>T (p.P30L), Cluster 6 (p.I236N, p.V237G, p.M239L), g.1683G>T (p.V281L), g.1994C>T (p.Q318X), and g.2108C>T (p.R356W). Different large deletions including all exonic parts of CYP21A2 account for 20-25% of 21-OH deficient cases in most populations (Rabbani et al, 2011). As a consequence of the pseudogene existence, various combinations of gene deletions, duplication and conversions which may occur on the basis of different breakpoints complicate genetic mutation analysis (Rabbani et al, 2011).

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 (Krone et al, 2005). Due to the difficulties in CYP21A2 mutation detection, several different methods such as the amplification-created restriction site approach (Lee et al, 1998), multiplex mini-sequencing (Krone et al, 2002), and direct gene sequencing (Malikova et al, 2012), have been applied to CAH genotyping. Chromosomal rearrangements have been investigated by Southern blotting (Concolino et al, 2009), quantitative real-time PCR (Parajes et al, 2007) and multiplex ligation-dependent probe amplification (MLPA) (Concolino et al, 2009). Direct sequencing in combination with MLPA offers the highest diagnostic information, however, being very time-consuming and costly, does not represent an attractive second-tier test for newborn screening programs at this stage (Malikova et al, 2012).

The CAH Strip Assay provides a fast (7 hs) and reliable protocol to identify common CYP21A2 mutations. Only small amounts of DNA are required, allowing analysis from dried blood spot punches

(Németh et al, 2012).

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.

Other aim of this study was to detect the copy number of the CYP21A2 gene to complete the genetic diagnosis profile for CAH patients (Mutations and copy number). Genetic investigations were done using real-time polymerase chain reaction (RT-qPCR) in Molecular Biology Research and Diagnostics Unit (MRDU) Faculty of Medicine (Kasr Alainy Hospital), Cairo University as a part of science and Technology Development Fund (STDF) project.

In the present study, the mean age of the studied patients was 6.2±3.2 years at time of sample collection. By karyotyping (definitive sex), females represented 69.7 % of the cases, while males represented 30.3%, although CAH is an autosomal recessive pathology and should occur equally in both genders.

Similarly, Pezzuti et al, (2014) considered that diagnosis of females more than males (4:1) is an indication for unrecognized deaths due to salt losing crisis in male children. Also this was similar to a study done by Marumudi et al, (2012) that included 62 patients, 56 were females and 6 were males. They suggested that this may be due to most of male children miss the correct diagnosis during infancy until or unless precocious puberty occurs.

Also, Yadav et al, (2015) observed female prevalence among the children with CAH (1 male : 12 female) and supposed that this might be due to the lack of neonatal screening, which could enable earlier diagnosis and reduce the number of deaths. On the other hand, Gidlof et al, (2013) found that the male : female ratio among CAH patients in Sweden was nearly 1:1 where neonatal screening for CAH was mandatory.

In the present study, Consanguity was observed in 132 cases (70.2%) thus highlighting the effect of this community based tradition on the prevalence of autosomal recessive disorders.

Shawky et al, (2011) found that the rate of consanguinity was 35.3% in Egypt, and they suggested that the high prevalence of consanguinity is a risk factor in DSD (Disorders of Sex Development) cases including CAH (the commonest type of DSD) due to accumulation of deleterious genes in families. In Iranian population, Rabbani et al, (2012) also found a high consanguinity rate (88.6%) were due to first-cousin marriages that urged them to perform their study to provide antenatal diagnosis, carrier detection and genetic counseling for afflicted families.

In contrast, Firdevs et al, (2009) reported consanguinity only in15 (28%) out of 52 families in Turkey ,also Mahrukh et al, (2016) reported consanguinity in 40% in a group of CAH indian patients. Also, Kolahdouz et al, (2016), reported only 23% consanguinity among the CAH patients in Iran, this difference from our study could be due to the restriction of non-classical phenotype and also because consanguinity differs among cultures, while Egypt is known to have high rate for consanguinity.

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 (Othman et al, 2014).

Similarly, Larissa et al, (2013) stated that at time of diagnosis, all of the patients had hyponatremia and hyperkalemia. Also, Firdevs et al, (2009) 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) 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, (2013) and Wedell et al, (2011).

Also, Larissa et al, (2013) stated that at time of diagnosis, all of the patients had high basal levels of 17- OHP (>50 ng/mL), Mahrukh et al, (2016) high basal 17OHP was observed in 94% of the patients. Kolahdouz et al, (2016) concluded that high level of 17OHP cannot recognize SW from SV cases and concluded that molecular tests for accurate diagnosis of CAH and type of disease are required.

In the current study, salt wasting clinical presentation was seen in 76.6 % of the cases, while simple virilizing clinical presentation was seen in 23.4 % (SW:SV ratio is 3.2:1). Similarly, Rabanni et al, (2012) reported that 81.8% and 18.2% of his patients were SW and SV respectively (SW:SV ratio is 4.5:1). Also in Katia et al, (2016) found out of 93 patients under study, 66 (71%) were affected with SW form, and 27 (29%) were affected with SV form. Also, Othman et al, (2014) reported that SW type accounted for about 75% of cases with classic CAH while SV type account for 25% (SW: SV ratio is 3:1).

In contrast, Firdevs et al, (2009) found that out of 56 patients, 26 (47%) were diagnosed as having SW form, 24(43%) as having SV form and 6 (10%) as having NC form. This difference from our study could be due to more diversity in clinical forms. Also, Eunice et al, (2012) studied CYP21A2 mutations is a group of 62 Indian CAH patients, 50 (80.6%) were simple virilizers and 12 (19.3%) were salt wasters. This difference from our study could be due to change of incidence of CAH clinical types among populations.

In the current study, among the female group, 9.2% were positive for hirsutism and 95.4 % had atypical genitalia. Similarly, Torres and co-workers, (2003) reported that 100% of the female patients had atypical genitalia. Also, Al-Maghribi, (2007) stated that among 39 females with CAH, 27 (69.2%) had developmental anomalies of the external genitalia. Also,Mahrukh et al, (2016) reported ambiguous genitalia in 70% and Hirsutism in 40% of females under study.

In the current study 21.1% of the studied male group had precocious puberty. This small percent could be due to the lack of the non-classical form of CAH in the current study, which manifests mainly by precocious puberty.

Similarly, Khan et al, (2002) reported that 14% of the males under study had precocious puberty and Mahrukh et al, (2016) in 8 cases (16%). Also, Sarar et al, (2015), reported that 30% of the males under study had precocious puberty. On the other hand, Yan-kun et al, (2015) reported that all the patients had precocious puberty and this is because the study was restricted to adult male patients who suffer from testicular tumors as consequence to uncontrolled CAH.
In the present study, Concerning 8bp deletion mutation, out of 126 patients who were negative for 8bp deletion mutation, 94 patients (65.3%) were salt wasting and 32 patients (72.7%) were simple virilizing. While 41 patients who were positive for 8bp deletion, 30 (20.8%) were salt wasting (10 heterozygous & 20 homozygous) and 11 (25%) were simple virilizing. On the other hand, 21 patients who had no gene, 20 (13.9%) were salt wasting and 1 (2.3%) were simple virilizing .This difference was statistically significant ($P = 0.004$).

Mornet et al, (1991) stated that 8bp deletion mutation is associated with SW phenotype. Similarly, Anca et al, (2011) stated that the most

frequent mutation in Romanian patients with 21OHD was I2G (29 of 66 alleles; 43.9%), followed by deletions (11 of 66 alleles; 16.7%), I172N and a triple mutation (P30L+I2G+del8bp) (each 8 of 66 alleles; 12.1%), P30L (5 of 66 alleles; 7.6%), R356W (1 of 66 alleles; 1.5%), and three double mutations. A total of 33.3% of patients (11 of 33) had a homozygous genotype, whereas 66.6% (22 of 33) were compound heterozygotes.

Identification of the genotype could be a guide in planning a strategy for prenatal treatment and for newborn screening programs. The relationship between genotype and phenotype may also be of value in evaluating response to treatment (Hughes, 2002). Thus, a health care policy program based on molecular genetics draws the only essential way to decrease the incidence of CAH in our country.

In the current study, the copy number results detected by Real-time PCR were as follow; 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.

Copy number variations (CNVs) have been described for the RCCX locus: the presence of two RCCX modules is the standard and the presence of one, three or even four modules are rare arrangements (Parajes et al, 2008).

Parajes et al, (2007) studied CYP21A2 copy number based on real-time PCR and gene copy assessment detects homozygous and heterozygous CYP21A2 gene deletions, CYP21A1P/CYP21A2 chimeric genes, and gene duplications. Also, the study found that the efficiency rates for the CYP21A2 gene with 3, 2, and 1 gene copy were 81%, 84%, and 99%, respectively.

The trimodular copy number organization accounts for only 14% of the chromosomes in population studies and the majority carry two copies of the CYP21A1P pseudogene and one copy of the CYP21A2 gene. The trimodular haplotype has also been described with two copies of the CYP21A2 gene and one copy of the CYP21A1P pseudogene. This latter haplotype has been described rarely in some carriers of the p.Gln318X mutation and chimeric CYP21A1P/CYP21A2 genes (Blanchong et al, 2000).

In contrast , Parajes et al, 2008, have shown a high frequency of this trimodular copy number organization arrangement, (7%) of studied individuals. The specific amplification and sequencing of each duplicated gene localized the p.Gln318X mutation at the CYP21A2 gene next to TNXB gene and it showed a wild-type CYP21A2 at the 39UTR of TNXA pseudogene.

Thus, PCR approaches based on the specific amplification of CYP21A2 like genes next to TNXB would fail in the detection of the wild-type gene and would misdiagnose these duplicated alleles as pathologic(Lee et al, 2004).

The duplicated CYP21A2 alleles found bear one inactivated gene due to the severe mutations and a wild-type gene which abolishes the pathological effect of the mutation (Baumgartner-Parzer et al, 2007).

Hence, an awareness of the presence of these types of gene duplications is important if a misdiagnosis is to be avoided. A possible misdiagnosis could be the erroneous identification of a wild type allele as pathological. This is critical for the molecular diagnosis of 21OHD, as well as for prenatal testing and for genetic counseling (Parajes et al, 2008).

In this study, the frequency of 8bp deletion mutation that was diagnosed by strip hybridization test showed the heterogenous pattern (mutation in one allele) in 10.1% of the studied patients.

Similarly, Parajes et al, 2008, demonstrated higher carrier frequency 15.3%. It is also similar to data obtained from genotyping in New Zealanders (White et al, 2000).

Nevertheless, it is much lower than the frequency described for Middle European population (5.5%) (Baumgartner-Parzeret al, 2005).

Also, in this study, the frequency of the wild pattern (No deletion) mutation of CYP21A2 gene that was diagnosed by strip hybridization test was 67.0%. however, Parajes et al, 2008 demonstrated that the high frequency of novel genetic variants require the importance of sequencing the whole CYP21A2 gene before it can be considered as wild-type.

Molecular analysis of the CYP21A2 gene has been proposed as an alternate second-tier testing approach to reduce the high rate of false positive results of 17OHP in neonatal screening for CAH (Christopher et al, 2014).

A second concern about using a molecular second-tier approach is that CAH is a critical disorder, and turn-around time for results is important to detect infants with CAH prior to a salt-wasting crisis. (Christopher et al, 2014).

The current study successively assessed the strip hybridization procedure in terms of its specificity ,sensitivity and overall accuracy compared to RT-qPCR as a more reliable method, concerning 8bp deletion mutations in CYP21A2 in Egyptian children.

According to Carrera et al, (1997), RT-qPCR is a rapid, reliable and non-radioactive method for detecting any mutation involving single base changes or small deletions. In all the samples analyzed by RT-qPCR, there was excellent agreement with the results obtained by sequencing. The major problem with RT-qPCR is to differentiate between failed PCR and homozygosity. This was resolved by including external control DNA samples from subjects who had been previously genotyped, either homozygous or heterozygous. The control PCR reactions were set up in parallel with the DNA samples to be tested and amplifications were run simultaneously (Carrera et al, 1997).

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 (Lee et al, 2005).

Also, Christopher et al, (2014) reported that RT-qPCR has demonstrated the specificity required for the development of a second-tier molecular assay with a low false-positive and false-negative rate that could serve as the basis for an appropriate CAH neonatal screening.

Wedell and Luthman, (1993) stated that among RT-qPCR disadvantages being a multi-step process that is subjected to lots of human errors, for example, obtaining false positive results if amplification of the active CYP21A2 gene only without the pseudogene was not done properly.

Also, Németh et al, (2012) reported that the complexity of the chromosomal region containing CYP21A2 and the degree of similarity between this gene and the CYP21A1P pseudogene require a highly-specific amplification strategy to avoid false positive and false negative results. In the present study, we added a step for specific amplification of the active gene to overcome this problem.

Also Dianne et al, (2005) reported that RT-qPCR results may be

difficult to interpret, unless the mutations identified can be confidently assigned to the CYP21A2 or pseudogene loci.

In a study done for strip hybridization assay for rapid detection of common CYP21A2 mutations in dried blood spots from newborns with elevated 17-OH progesterone, Németh et al, (2012) concluded that CAH Strip assay provides a fast (7 h) and reliable protocol to identify common CYP21A2 mutations. Only small amounts of DNA (≥ 1 ng) are required, allowing analysis from dried blood spot punches. If implemented as a second-tier test in CAH newborn screening programs, a significant reduction of recall rates could be achievable.

However, Németh et al, (2012) found out that Strip hybridization assay approach is limited by its inability to identify chimeric CYP21A1P/CYP21A2 genes with junction sites downstream of cluster E6 as well as complete CYP21A2 deletions in samples that are heterozygous for these lesions.

Consequently, hemizygosity for a point mutation cannot be discriminated from homozygosity whenever such a large rearrangement is present on the other chromosome. This deficit, however, is alleviated by the fact that the clinical phenotype of CAH is typically linked to the less severe mutation, and thus such situations will ultimately lead to the same diagnosis.

Also, Németh et al, (2012) stated that all eleven point mutations and 51% of large deletions/conversions could be unambiguously identified when compared to reference methods (DNA sequencing, MLPA). After exclusion of rare mutations 6.4% not covered by the Strip Assay, the overall detection rate was 85%.

Ultimately, the uncertainty that may arise due to undetected deletions/conversions can be overcome easily by using the reverse-hybridization technique in combination with methods determining gene copy numbers, such as the quantitative real-time PCR approach of Parajes et al, (2007). When adding this method to Strip Assay analysis, the detection rate of large deletions and conversions could be increased from 51% to 100%.

REFERENCES

1. Abou El-Ella, Maha A Tawfik, Wafaa M Abo El-Fotoh,…… et al. (2016): "Genetic evaluation of children with ambiguous genitalia." Menoufia medical journal.; 29 : 79-88.
2. Acar E, et al. (2009): Optimization and validation studies of the MentypeR Argus X-8 kit for paternity cases. Forensic Sci Int: Genet Suppl.; 2: 47–48.
3. Akolekar R, et al. (2015): Procedure-related risk of miscarriage following amniocentesis and chorionic villus sampling: a systematic review and meta-analysis. Ultrasound in Obstetrics & Gynecology.; 45(1): 16-26.
4. Al Swailem M.M. (2016) : Molecular genetics of non 21α-hydroxylase congenital adrenal hyperplasia and 5α-reductase deficiency in Saudi Arabia, Alfaisal University (Saudi Arabia).
5. Allen D, Hoffmann G, Fitzpatrick P, Laessig R, Maby S. and Slyper A. (1997): Improved precision of newborn screening for congenital adrenal hyperplasia using weight-adjusted criteria for 17-hydroxyprogesterone levels. J Pediatr.; 130 (1) : 128–33.
6. Al-Maghribi H. (2007): Congenital adrenal hyperplasia: problems with developmental anomalies of the external genitalia and sex assignment, Saudi J Kidney Dis Transpl. Sep.; 18(3):405-13.
7. Anca G.S, Matthias M.W, Paula G.S, Susanne C, Udo H. and Egbert S. (2011): 21-Hydroxylase and 11β-Hydroxylase Mutations in Romanian Patients with Classic Congenital Adrenal Hyperplasia, First Clinic of Internal Medicine, Department of Endocrinology, Johannes Gutenberg University, Langenbeckstr.; April 29:54-59.
8. Andermann A, et al. (2008) : "Revisiting Wilson and Jungner in the genomic age: a review of screening criteria over the past 40 years." Bulletin of the World Health Organization.; 86(4): 317-19.
9. Araújo M.B. and New M. (2007): Ensemble forecasting of species distributions. Trends in ecology & evolution.; 22(1): 42-47.
10. Aslih N, and Ellenbogen A. (2014): "Congenital Adrenal Hyperplasia, the Origin of Combined Infertility: A Case Report and a Review of Literature." Reprod Syst Sex Disord.; 3: 2.
11. Attardi B.J, Zeleznik A, Simhan H, Chiao J.P, Mattison D.R. and Caritis S.N. (2007): Comparison of progesterone and glucocorticoid receptor binding and stimulation of gene expression by progesterone, 17-alpha hydroxyprogesterone caproate and related progestins. Am. J. Obstet. Gynecol.; 197 (6): 599–97.
12. Balsamo A, Alessandro C, Lilia B, Michela B, Federico B, Monia G, Milva B, Alessandra C, Krissi K, and Emanuele C. (2003): "CYP21 genotype, adult height, and pubertal development in 55 patients treated for 21-hydroxylase deficiency." The Journal of Clinical Endocrinology & Metabolism 88.; no. 12 : 5680-88.
13. Barra C.B, et al. (2012) : Neonatal screening for congenital adrenal hyperplasia. Revista da Associação Médica Brasileira (English Edition).; 58(4): 459-64.
14. Baş F, Hülya K, Feyza D, Oya U, Hülya G, Memnune Y.A, Fatmahan A, et al. (2009): "CYP21A2 Gene Mutations in Congenital Adrenal Hyperplasia: Genotype–phenotype correlation in Turkish children." Journal of clinical research in pediatric endocrinology 1.; no. 3: 116.
15. Bauland C.G, et al. (2012): Similar risk for hemangiomas after amniocentesis and transabdominal chorionic villus sampling. Journal of Obstetrics and Gynaecology Research.; 38(2): 371-75.
16. Benn P, et al. (2013): Position statement from the Aneuploidy Screening Committee on behalf of the Board of the International Society for Prenatal Diagnosis. Prenatal diagnosis .; 33(7): 622-29.
17. Binay C, Enver S, Oguz C, Zafer Y, Ozden K, and Sevilhan A. (2014): Prevalence of nonclassic congenital adrenal hyperplasia in Turkish children presenting with premature pubarche, hirsutism, or oligomenorrhoea. International Journal of Endocrinology .; 95(12):1635–41.
18. Bizzarri C, Crea F, Marini R, Benevento D, Porzio O, Ravà L. and Cappa M. (2012): Clinical features suggestive of non-classical 21-hydroxylase deficiency in children presenting with precocious pubarche. Journal of Pediatric Endocrinology and Metabolism.; 25: 1059- 64.
19. Blanchong C.A, Zhou B, Rupert K.L, Chung E.K, Jones K.N, Sotos J.F, et al. (2000): Deficiencies of human complement component C4A and C4B and heterozygosity in length variants of RP-C4-CYP21-TNX (RCCX) modules in Caucasians: the load of RCCX genetic diversity on major histocompatibility complex-associated disease. Journal of Experimental Medicine.; 191(12): 2183-96.
20. Bustin S.A. (2000): Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol.; 25 (2): 169–93.
21. Cargill M, Altshuler D, Ireland J, Sklar P, Ardlie K, Patil N, Lane C.R, Lim E.P, Kalayanaraman N, Nemesh J, Ziaugra L, Friedland L, Rolfe A, Warrington J, Lipshutz R, Daley G. and Lander E. (1999): Characterization of single-nucleotide polymorphisms in coding regions of human genes. Nat Genet.; 22: 231–38.
22. Carrera P, Barbieri A. M, Ferrari M, Righetti P.G. & Perego M. (1997): Rapid detection of 21-hydroxylase deficiency mutations by allele-specific in vitro amplification and capillary zone electrophoresis. Clinical chemistry.; 43(11): 2121-27.
23. Chen W, Xu Z, Sullivan A, Finkielstain G. P, Van Ryzin C, Merke D.P. and McDonnell N.B. (2012): Junction site analysis of chimeric CYP21A1P/CYP21A2 genes in 21-hydroxylase deficiency. Clinical chemistry.; 58(2): 421-30.
24. Chew S. and Leslie D. (2006): Clinical endocrinology and diabetes: an illustrated colour text, Elsevier Health Sciences.
25. Choi J.H, Gu H.K. and Han W.Y. (2016): "Recent advances in biochemical and molecular analysis of congenital adrenal hyperplasia due to 21-hydroxylase deficiency." Annals of pediatric endocrinology & metabolism.; 21, no. 1: 1-6.
26. Christopher N.G, Suzanne K.C, Daniel P.T, Lisa M. K, Dorothy S. and Patricia W.M. (2014): Novel method to characterize CYP21A2 in Florida patients with congenital adrenal hyperplasia and commercially available cell lines.; volume 1: 312-23.
27. Clayton P.E, Miller W.L, Oberfield S.E, Ritzén E.M, Sippell W.G. and Speiser P.W. (2002): Consensus statement on 21-hydroxylase deficiency from the European Society for Paediatric Endocrinology and the Lawson Wilkins Pediatric Endocrine Society. J Clin Endocrinol Metab.; 87(9): 4048–53.
28. Coeli-Lacchini F.B, Turatti W, Elias P.C.L, Elias L.L.K, Martinelli C.E, Moreira A.C, et al. (2013): A rational, non-radioactive strategy for the molecular diagnosis of congenital adrenal hyperplasia due to 21-hydroxylase deficiency.; 526(2): 239-45.
29. Concolino P, Mello E, Toscano V, Ameglio F, Zuppi C. and Capoluongo E. (2009): Multiplex ligation-dependent probe amplification (MLPA) assay for the detection of CYP21A2 gene deletions/duplications in congenital adrenal hyperplasia: first technical report. Clin Chim Acta.; 402: 164–70.
30. Concolino P, Mello E, Zuppi C. and Capoluongo E. (2010): Molecular diagnosis of congenital adrenal hyperplasia due to 21-hydroxylase deficiency: an update of new CYP21A2 mutations. Clin Chem Lab Med.; 48(8): 1057-62.
31. Cradic K.W, Murphy S.J, Sikkink R.A, Neuhauser C, Vasmatzis G. and Grebe S.K. (2015): Clinical validation of a haplotyping method with next-generation sequencing. Clinical chemistry.; 61(2) : 430-31.
32. Day D, Speiser P, White P. and Barany F. (1995): Detection of steroid 21-hydroxylase alleles using gene-specific PCR and a multiplexed ligation detection reaction. Genomics.; 29: 152– 62.
33. Dehghani M, et al. (2014): A newborn with ambiguous genitalia and a complex X; Y rearrangement. Iranian journal of reproductive medicine.; 12(5): 351.
34. Delle P.L, et al. (2015): 150 years of congenital adrenal hyperplasia: translation and commentary of De Crecchio's classic paper from 1865. Endocrinology.; 156(4): 1210-17.
35. Dianne K.K, Joy B.R, Reno U.A, Michele M.E, Robert C.W, Maria I.N, Jon M.N. and Raymond G.F. (2005): Validation and Clinical Application of a Locus-Specific Polymerase Chain Reaction- and Minisequencing-Based Assay for Congenital Adrenal Hyperplasia (21-Hydroxylase Deficiency), May.; 7(2): 236–46.
36. Dimitri P. and Wales J.K.H. (2012): Growth and Puberty In: Lissauer T, Clayden G (editors), Illustrated Textbook of Paediatric Mosby; 4th edition.; 11:178-99.