Indexed by : DOAJ Google Crossref Dimension sinta GARUDA

J.Biomed.Transl.Res
ISSN: 2503-2178

JOURNAL OF BIOMEDICINE AND TRANSLATIONAL RESEARCH

Available online at JBTR website: https://jbtr.fk.undip.ac.id

Original Research Article

Rapid PCR-RFLP Screening of *CYP21A2* Variants for Salt-Wasting Congenital Adrenal Hyperplasia (CAH): Bridging Molecular Diagnostics in a Low-Resource Setting

Irene Astrid Larasati[1], Nani Maharani[2,4], Agustini Utari[3,4], Tri Indah Winarni[4*]

[1]*Doctor of Medicine and Health Study Program, Faculty of Medicine, Universitas Diponegoro, Indonesia*
[2]*Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Diponegoro, Indonesia*
[3]*Department of Pediatrics, Division of Pediatric Endocrinology, Faculty of Medicine, Universitas Diponegoro, Indonesia*
[4]*Center for Biomedical Research (CEBIOR), Faculty of Medicine, Universitas Diponegoro, Indonesia*

Article Info
History
Received: 18 Nov 2025
Accepted: 13 Feb 2026
Available: 30 Apr 2026

Abstract

Background: The 21-hydroxylase deficiency (21OHD) accounted for the majority of autosomal recessive inherited congenital adrenal hyperplasia (CAH) cases. A genetic test could offer a more thorough diagnostic approach because of the wide range of disease severity, but it was challenging in a resource-limited setting.

Objective: To apply a simple and cost-effective rapid molecular screening for detecting the most common *CYP21A2* variants causing salt-wasting (SW) CAH.

Methods: DNA extraction was conducted using a salting out method from a venous blood sample taken from 49 SW CAH patients without prior CAH-specific genotyping. A PCR-RFLP approach was utilized using primer sets specifically designed to anneal to the *CYP21A2*. Specific restriction enzymes were selected to cleave the DNA sequence, differentiating the wild and mutant type, i.e, the p.Arg357Trp, p.Gln318Ter, IVS2-13A/C>G, and exon 6 cluster. Samples from a previous cohort that carried those variants detected by DNA sequencing were used as positive controls.

Results: Only 6.1% of 49 patients were found to have the p.Gln318Ter variant. Both the p.Arg357Trp and exon 6 cluster carried inconclusive results. The IVS2-13A/C>G variant displayed limited assay robustness among 27 individuals.

Conclusion: The PCR-RFLP was an advantageous screening method for identifying p.Gln318Ter variants in a low-resource setting. Nevertheless, the use of multiplex ligation-probe dependent amplification (MLPA) and Sanger sequencing offered a comprehensive analysis to discover novel variants that could help with patients' diagnosis and treatment.

Keywords: *Congenital adrenal hyperplasia; CYP21A2; PCR; RFLP; developing country.*
Permalink/ DOI: https://doi.org/10.14710/jbtr.v12i1.30107

INTRODUCTION

Congenital adrenal hyperplasia (CAH) (CAH; OMIM #201910) is a group of genetic conditions characterized by an enzymatic defect in adrenal steroidogenesis. In 95% of cases, the condition is caused by pathogenic variants in the *CYP21A2* gene resulting in 21-hydroxylase deficiency (21OHD) leading to cortisol, and aldosterone deficiency in the most severe type, and excessive androgen production.[1] Phenotypically, 21OHD encompasses a spectrum of the

*Corresponding author:
E-mail: triwinarni@lecturer.undip.ac.id
(Tri Indah Winarni)

most severe salt-wasting (SW) to the least severe non-classic (NC) type. The incidence rate of classic CAH is 1 to 10,000 to 18,000 live birth according to the newborn screening (NBS) data.[2]

According to the CAH Registry by the Indonesian Pediatric Society, there was a 391.3% increase in the number of CAH cases in Indonesia from 2009 to 2020, with females dominating male cases (female-to-male ratio=303:136).[3] Many CAH individuals were late-diagnosed due to the absence of an NBS program, which had barely been started in early 2025 in Indonesia. Individuals with CAH were referred to a pediatric endocrinologist only after frequent episodes of vomiting, dehydration, weight faltering, and weight loss without improvement after treatment, and the presence of virilization aided in the diagnosis of female CAH. However, the number of males with CAH was underrepresented, which was most likely caused by the absence of genital ambiguity and misdiagnosis of adrenal crisis as gastroenteritis.[4,5] Timely diagnosis is crucial to prevent the lethality of the adrenal crisis, especially in the most severe SW form. With the beginning of CAH NBS in Indonesia, medical laboratories should improve their capacity and capability, particularly in the measurement of 17-hydroxyprogesterone (17OHP) and genotyping of the *CYP21A2*.

The *CYP21A2* (GenBank NG_007941.3; Ensembl ENSG00000231852; AllianceGenome HGNC:2600) has a highly homologous pseudogene, the *CYP21A1P*, in which both are located in a human leukocyte antigen (HLA) class III on the short arm of chromosome 6 (6p21.3), where a high frequency of gene recombination occurs.[6] Hence, genotyping the gene, interpreting the results, and providing a comprehensive genetic counseling required a thorough knowledge of the genetics of CAH. The genetic test could provide a reliable diagnosis, particularly in terms of inconclusive results after cosyntropin stimulation test, and is especially valuable in SW CAH, in which prompt recognition of molecular variants informs prognosis, anticipates adrenal crisis, and allows timely genetic counseling, including carrier detection, and family planning.[7] Among the nine most common *CYP21A1P*-derived variants, the 8 bp deletion in exon 3 (E3Δ8 bp), the exon 6 cluster point variants (p.[Ile236Asn; Val237Glu; Met239Lys]), p.Gln318Ter, p.Arg357Trp, and the intron 2 splice (IVS2-13A/C>G) variant were associated with SW CAH.[8] In a previous study on 50 individuals with 21OHD in our center, the exon 6 cluster point variants, p.Gln318Ter, p.Arg357Trp, and the intron 2 splice variant were found in 1, 3, 44, and 7 alleles, respectively. The deletion and E3Δ8 bp were not found in the cohorts (Utari et al, unpublished data). Due to a lack of resources, the *CYP21A2* genetic test using multiplex ligation-probe dependent amplification (MLPA) and Sanger sequencing could not be performed in Indonesia. Therefore, we aimed to conduct a cost-effective, simple, and reliable method suitable for any laboratory with conventional PCR and gel electrophoresis using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) in the leading CAH center in Indonesia focusing on the detection of the prevalent point variants causing the most severe SW CAH, including the intron 2 splice, exon 6 cluster, p.Gln318Ter, and p.Arg357Trp. This method was suitable to be applied in a resource-limited setting and unveiled a possibility of *CYP21A2* genotyping as a molecular screening approach for most common variants, which may assist clinical decision-making following CAH NBS, especially when cosyntropin stimulation test result was inconclusive or when genetic counseling was indicated.

MATERIALS AND METHODS

This was a cross-sectional genetic study conducted in a genetics research laboratory in Semarang, Indonesia. The study was conducted from November 2023 to June 2024.

The participants of this study were individuals with CAH without prior CAH-specific genetic test who were diagnosed by pediatric endocrinologists at a tertiary and secondary hospital in Semarang, Indonesia. The diagnosis of CAH was made based on the clinical characteristics, i.e., vomiting, diarrhea, dehydration, hyperpigmentation, and virilization in females, and biochemical measurement of 17OHP and electrolytes, i.e., sodium and potassium. Karyotyping was performed to confirm sex chromosomes in individuals presenting with genital ambiguity. Participants were approached during their routine follow-up and offered their participation in the study. Parents or caregivers of younger participants (<18 years) provided written consent for participation in the study after receiving and understanding the information given by the research team.

The participant's blood was drawn from a cubital venous vessel by trained laboratory technicians. The blood was stored in an EDTA tube at -80°C. A salting-out method was utilized to extract the DNA. DNA concentration and purity were measured using NanoVue Plus Spectrophotometer (GE Healthcare Technologies, Inc., Chicago, IL) by calculating the DNA samples' absorbance at 260 nanometers (A260) and the ratio of absorbance at 260 and 280 nanometers (A260/A280), respectively. A A260/280 ratio at ≥1.80 was considered as the value of pure DNA.

Positive and negative controls were selected from previous cohorts in our center with known pathogenic variants, also considering their hetero-/homozygosity states. The controls for IVS2-13A/C>G, p.Gln318Ter, and exon 6 cluster variants were heterozygous for each variant, while the control for p.Arg357Trp was homozygous for this variant.

The PCR process was carried out using the GeneAmp PCR System 9700 (Thermo Fisher Scientific Inc., Waltham, MA). Two rounds PCR amplification was utilized for the IVS2-13A/C>G variant, in which the first round was targeted to specifically amplify the exon 1, exon 2, and the intron 2 site of the *CYP21A2* gene using a set of LR-F/B2 primers, and the second round was aimed to amplify the intron 2 using a set of 7/8 primers, producing fragment sizes of 1,118 bp and 155 bp, respectively. The other variants, i.e., the Exon 6 cluster (E6 cluster; a compound missense variant named p.[Ile236Asn; Val237Glu; Met239Lys], p.Gln318Ter, and p.Arg357Trp, were amplified using a

set of long-range PCR primers, i.e., C1/LR-R, producing a fragment size of 2,610 bp. The site of the primer's annealing was displayed in Figure 1. Some of the primers used in the study were based on a previous study by Zhang et al.[10]

The mixture of each sample for the first round of PCR included 8.2 µL of H_2O, 10 µL of PCR mix, 0.4 µL of forward primer, 0.4 µL of reverse primer, and 2 µL of DNA (100 ng). The PCR settings for the IVS2-13A/C>G variant were 1 cycle of initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, and concluded with a final extension at 72°C for 5 minutes. The PCR settings for the E6 cluster, p.Gln318Ter, and p.Arg357Trp were 1 cycle of initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 59.3°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes.

The mixture for the second round of PCR comprised 8.2 µL of H_2O, 10 µL of PCR mix, 0.4 µL of forward primer (primer 7), 0.4 µL of reverse primer (primer 8), and 1 µL of DNA (100 ng). The PCR settings for this process involved 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds.

A product check was conducted after each round of PCR using the 1.5% and 2.5% agarose gel electrophoresis for sequences larger and smaller than 1000 bp, respectively. Gel electrophoresis was run for 35 minutes at 120 V. Although PCR product clean-up was not performed prior to RFLP, a PCR product check ensured its specificity and quality by visualizing the amplified predetermined fragments before proceeding to RFLP.

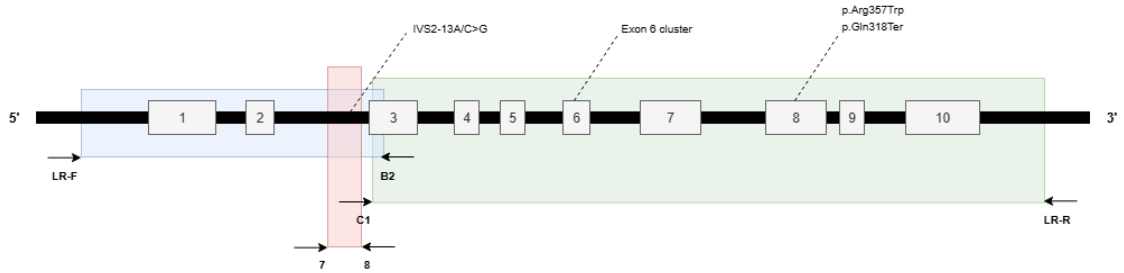
Prior to the RFLP analysis, an in-silico analysis using a restriction analyzer tool (https://molbiotools.com/restrictionanalyzer.php) was conducted utilizing the *CYP21A2* (NG_007941.3) sequence retrieved from the human genome assembly GRCh38/hg38 (unpublished data) to analyze the restriction sites and to visualize the digested bands.

The reaction mixture for RFLP was comprised of 0.5 µL of buffer Tango, 0.5 µL of 10x PCR buffer, 0.5 µL of restriction enzyme, 8.5 µL of H2O, and 10 µL of PCR product. Digestion process was conducted for 2 hours at a temperature of 37°C. To visualize the fragments, a 1.5% or 2.5% agarose gel electrophoresis was operated for 40 minutes at 110 V to effectively separate the DNA fragments based on their length. Each restriction enzyme's specific site was displayed in Table 1.

Ethical approval was obtained before the commencement of the study (No. 072/EC/KEPK/FK-UNDIP/II/2024). Data were presented descriptively as frequencies and percentages. No statistical analysis was conducted in the study.

RESULTS

All 49 CAH individuals included in the study were clinically diagnosed with SW type. The 46,XX karyotype was observed in 63.3% (31/49) individuals. The majority (37/49) was first referred to the center before age 1 year.

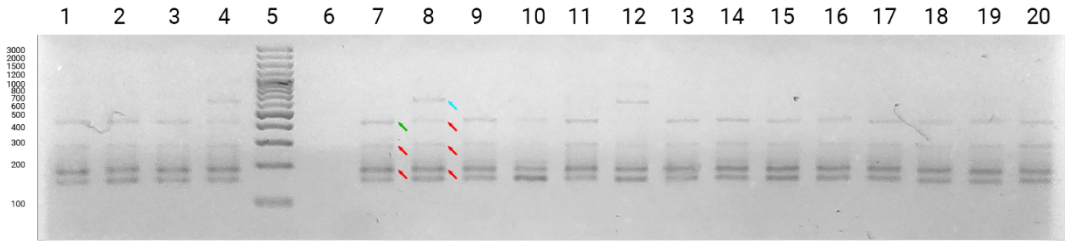


Right arrows: forward primers; left arrows: reverse primers; dash lines: annotated *CYP21A2* point variants; blue box: PCR fragment from the first round amplifying exon 1 and 2; red box: PCR fragment from the second round amplifying intron 2; green box: PCR fragment amplifying exon 3 to 10.

Figure 1. Annealing sites of PCR primers for the *CYP21A2*

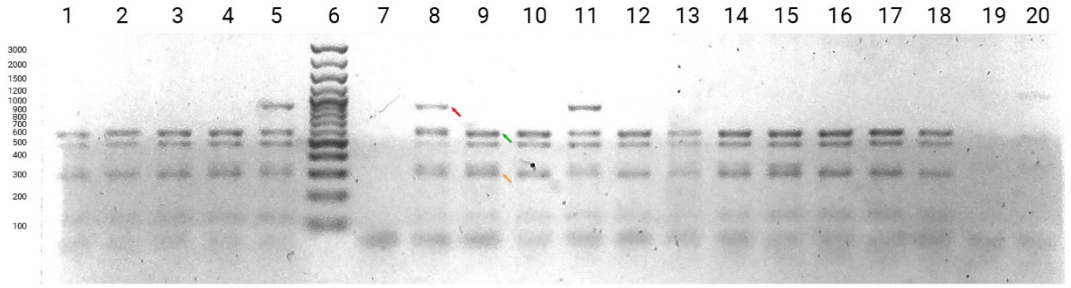
Table 1. *Restriction enzymes for digesting the CYP21A2 fragments causing SW CAH*

Variant	Restriction enzyme	Sequence Specificity	Fragment size (bp)	
			Wild-type	Mutant
IVS2-13A/C>G	Sau3AI	/GATC	156	133/23
p.Gln318Ter	PstI	CTGCA/G	567/298	865
p.Arg357Trp	AciI	CCGC(-3/-1)	189/30	219
p.[Ile236Asn; Val237Glu; Met239Lys]	DraIII	CACNNN/GTG	687/28	715



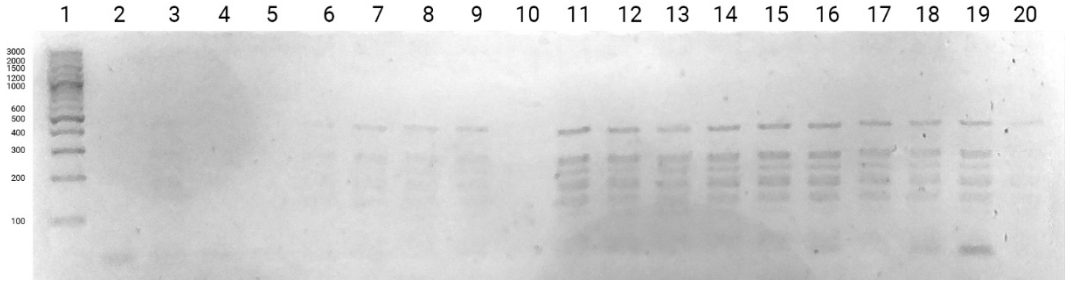
Marker: lane 5; non-template control: lane 6; positive control: lane 7; negative control: lane 8; positive results: lane 1–3, 9–11, 13–20; negative results: 4 and 12 (CAH 112 and 120). Red arrows show unknown fragments in both positive and negative controls, green arrow shows unknown in positive control, and blue arrow shows unknown fragment in negative control. Fragment sizes are pointed out on the side in bp.

Figure 2. *RFLP result of IVS2-13A/C>G*



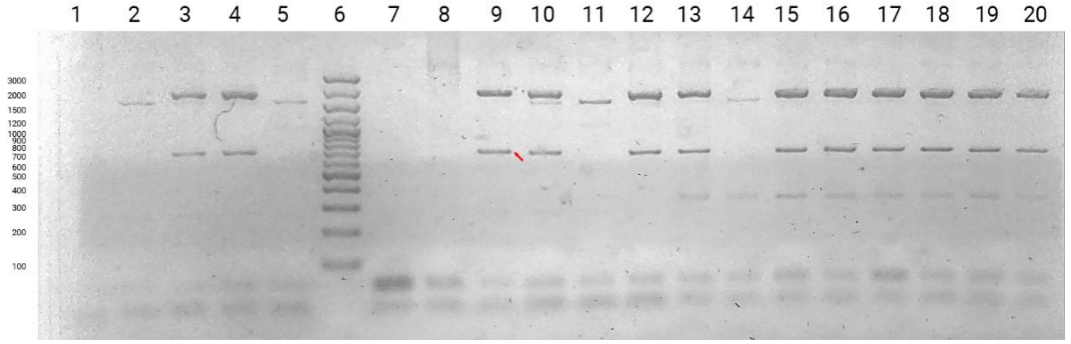
Marker: lane 6; non-template control: lane 7; positive control: lane 8; negative control: lane 9; positive results: lane 5 and 11 (CAH 079 and CAH 088); negative results: 1–4 (CAH 120, 121, 072, 077), 10 (CAH 086), 12–18 (CAH 094, 096, 100, 102, 103, 104, 105, 111); inconclusive: 19 and 20 (CAH 111 and 117). Red arrow shows fragment at 865 bp in the positive control, green and orange arrows show fragments at 567 and 298 bp, respectively. Fragment sizes are pointed out on the side in bp.

Figure 3. *RFLP Results of p.Gln318Ter*



Marker: lane 1; non-template control: lane 2; positive control: lane 3; negative control: lane 4; inconclusive: lane 5–20. Fragment sizes are pointed out on the side in bp.

Figure 4. *RFLP Results of p.Arg357Trp*



Marker: lane 6; non-template control: lane 7; positive control: lane 8; negative control: lane 9; inconclusive: 1–5, 10–20. Red arrow shows fragment at 719 bp in the negative control. Fragment sizes are pointed out on the side in bp.

Figure 5. *RFLP Results of p.[Ile236Asn; Val237Glu; Met239Lys] variant*

Figure 2 displayed the several digested DNA fragments after RFLP of the IVS2-13A/C>G variant. The positive control (lane 7) produced 4 DNA fragments at around 150 bp, 180 bp, 300 bp, and 450 bp, whereas the fifth fragment was visible in the negative control (lane 8) at around 650 bp.

Post-digested DNA products using the PstI enzyme visualized several fragments. The positive result showed an additional fragment at 825 bp, which was not present in the negative result. (See Figure 3)

Analysis of the post-digested DNA product using AciI of p.Arg357Trp displayed no band in either positive or negative controls. Several DNA samples showed fragments approximately 500 bp, 300 bp, 200 bp, and 100 bp. (See Figure 4)

There was no DNA fragment visualized on the positive control (number 2), although two fragments were visualized on the negative control (number 22). The samples exhibited different fragment sizes, as shown in Figure 5.

Detected Variants

The exon 6 cluster displayed absent amplification of expected fragment in the positive control. The controls for p.Arg257Trp exhibited inconsistent results, i.e., bands were sometimes present or absent, throughout analysis, limiting its interpretability. The IVS2-13A/C>G variant was assessed for technical feasibility in 27 individuals, however, positive results were found in 26 individuals demonstrating limited robustness in our setting. As a result, these variants were excluded from genotype-phenotype correlation of the full cohorts using PCR-RFLP approach.

Among the 49 patients analyzed in the study, the p.Gln318Ter variant was found in heterozygous state in three individuals. Thirty four (69.4%) individuals were found negative of this variant. Even though SW CAH diagnosis was established in all three individuals, genotype-phenotype correlation could not be performed due to the absence of conclusive results of the other targeted variants.

DISCUSSION

A rapid molecular analysis for detecting the four most common *CYP21A2* variants causing SW CAH was performed in the main CAH center in Indonesia. The analysis utilized sets of primers designed for long-range PCR, combined with the specific digestion enzyme used in the RFLP. The primers, i.e, B2 and C1, comprised the sequence absent in the pseudogene, such as the 8 bp deletion in exon 3 (E3Δ8bp; c.332_339del; p.Gly111fs; rs387906510), ensuring the amplification of the *CYP21A2*.[11] Nevertheless, the Del and E3Δ8bp could not be distinguished due to the location of both PCR primers in exon 3. A previous study conducted in 62 other Indonesian CAH patients displayed that the E3Δ8bp variant was found only in 1 allele, and the Del variant was found in none among 124 alleles analyzed.[9]

Table 2*. Genotype-phenotype frequency of Indonesian SW-CAH patients*

	Positive	Negative	No Band	Total
p.Gln318Ter	3 (6.1%)	34 (69.4%)	12 (24.5%)	49 (100%)
p.Arg357Trp[§]	N/A	N/A	N/A	N/A
		N/A	N/A	N/A
p.[Ile236Asn; Val237Glu; Met239Lys] [§]	N/A	N/A	N/A	N/A

[§]Uninterpretable due to the absent of DNA band in controls; N/A: Not available

Table 3. *Genotype-phenotype correlation of Indonesian SW-CAH patients*

Group	Variant	No. of Patients n (%)	Expected Phenotype	Expected Residual Enzymatic Activity
Null	p.Gln318Ter	3 (6.1)	SW	0%
	p.Arg357Trp[§]	N/A		
	p.[Ile236Asn; Val237Glu; Met239Lys] [§]	N/A		
A	N/A	N/A	N/A	N/A
B	N/A	0	N/A	N/A
C	N/A	0	N/A	N/A

[§]Uninterpretable due to the absent of DNA band in controls; N/A: Not available

Among the four-point pathogenic variants associated with SW CAH, the p.Gln318Ter variants were successfully identified because of larger-sized fragments visualized on the UV-illuminator. In contrast, interpretation of p.Arg357Trp was challenging due to the inconsistent appearance of DNA fragments of the positive and negative controls across different samples. Additionally, p.Arg357Trp could hardly be identified due to the insignificant fragment size differences between the wild and mutant types, i.e., 189 and 30 bp in the wild type and 219 bp in the mutant type, despite a higher agarose gel concentration at 2.5% and lower Voltage and shorter duration had been implemented. The application of the 100 bp + 3k marker in the study made the interpretation of DNA fragments smaller than 200 bp difficult. The approach applied relied solely on the visualization of DNA bands using a UV-illuminator. Conversely, previous studies utilizing a similar method successfully recognized the variant because of the use of Southern Blot and direct sequencing to confirm the deletions or gene conversion and base substitution, respectively.[10–12]

The exon 6 cluster yielded inconclusive results due to the absence of DNA fragments of the positive control after digestion by a specific restriction enzyme, DraIII. The unavailability of the product after the digestion by a restriction enzyme did not rule out the possibility of contamination. Preceding studies by Yokoyama et al.[11] and Zhang et al.[10] performed PCR product purification prior to the RFLP procedure. During the process, primers, reagents, and other contaminants that possibly could obstruct the subsequent process were removed.[13,14] Excess magnesium could chelate away the restriction enzyme, and frequently, the PCR reagents would inhibit the restriction enzymes. Hence, contamination prevention was necessary and justified.[15]

The IVS2-13A/C>G variant was found in 26 (96.3%) out of 27 individuals. However, the preceding study conducted in the same center displayed the p.Arg357Trp to be the most prevalent (41/62) variant and only a small number of individuals (7/62) had the intron 2 splice variant.[9] It could be possible that the high frequency of the intron 2 splice variant in the study was caused by technical issues during the second round PCR, because the digested product after RFLP showed various unforeseen fragments inconsistent with the expected 155 bp second round PCR product. We hypothesized the possibility of polymorphism in the Indonesian population, thus, the primers could not anneal in the intended site.[16] Therefore, another test, such as direct sequencing, should be conducted to confirm it.

The p.Gln318Ter variant was present in a heterozygous state in three individuals. Nevertheless, the presence of a pathogenic variant was inadequate to conclude it as a disease-causing variant, since CAH is inherited in autosomal recessive way. Therefore, we could not perform genotype-phenotype analysis in the study. In comparison, other severe *CYP21A2* variants were variably found in various population, such as the intron 2 splice was commonly found in the Chinese, Indian, and Cuban populations,[17–20], and the Del in Germans and Austrians, Dutch, Polish, and Chilean populations.[21–24] These variants resulted in less than 2% enzymatic activity, thus producing extremely low levels of cortisol and aldosterone. Detection of these variants could aid in the genetic counseling process, encompassing the phenotype prediction, course of CAH, carrier detection, family planning, and management of the condition.[25]

The diagnosis of CAH was confirmed by second-tier steroids panel and electrolytes measurement using LC-MS/MS following an elevated 17OHP level detected by NBS. However, this approach was not possible in Indonesia due to the unavailability of CAH NBS. CAH individuals in our study were diagnosed based on clinical characteristics and biochemical measurement of 17OHP and electrolytes. The establishment of PCR-RFLP presented as a rapid molecular screening of the most common *CYP21A2* variants for CAH diagnosis in Indonesia, especially in individuals past infancy, individuals under GC treatment following hospital admission due to adrenal crisis, or individuals with equivocal adrenocortical profile after cosyntropin stimulation test. Additionally, genotyping could provide the benefit of genetic counseling, including risk calculation, family planning, and heterozygote detection, because the latter do not require medical treatment. Early genetic counseling could aid the patient and the family to be more prepared to deal with and adjust to the course of CAH. A genetic counselor played an important role in assisting the patient in the journey of acceptance, adaptation, and connecting them to healthcare professionals and support groups.[26,27] Hence, patients and their families could benefit from enhanced risk perception, positive risk behaviors, better knowledge, and a sense of personal control.[28]

Due to its complexity, genotyping the *CYP21A2* required a combination of molecular analysis techniques to be able to detect complex structural rearrangements, copy number variations (CNVs), insertions/deletions, and single-nucleotide variants (SNVs). RFLP is a simple, specific, and cost-effective method to detect variations in the DNA sequence, including SNPs and in/del. This method was appropriate to be applied as an early genetic screening in a resource-limited setting, such as Indonesia.[29] By combining it with PCR using specifically designed primers, this procedure could yield high reproducibility.[30] Nevertheless, RFLP was time-consuming and labor-intensive. The negative results from the study might be attributable to the presence of *CYP21A2* variants that were not targeted in the assay, as RFLP could only detect variants that alter specific restriction sited. Consequently, this method was unsuitable for detecting novel variants.[31] This was a critical disadvantage in clinical settings where the identification of novel variants could influence diagnosis and treatment. Furthermore, RFLP required a large quantity and quality of DNA, which could be challenging to collect from specific sample types or in situations when there was a shortage of samples.[32] Considering that up to the present there had been more than 200 pathogenic variants known to cause CAH, there is a better and more advanced technique for *CYP21A2* genotyping, i.e., MLPA and Sanger

sequencing.[33] The integration of these methods allowed a more thorough understanding of *CYP21A2*.[34,35]

This study exhibited feasibility of performing rapid molecular screening of p.Gln318Ter variant causing SW CAH in a resource-limited setting. PCR product clean-up was not performed, however, verification of PCR product specificity was carefully measured using gel electrophoresis to ensure correct expected fragments were amplified. This approach was utilized to enhance feasibility in resource-limited setting without compromising the interpretability of RFLP results in the targeted variants. Polyacrylamide gel, different DNA ladders, and Sanger sequencing were not incorporated in the study to resolve small DNA fragments. These methods, in addition to more robust assay, such as MLPA, may be considered in future works to complement screening approach. Due to ethnic and region-dependent variability of *CYP21A2* variants, future investigations across diverse populations are warranted. Nevertheless, the present data provided valuable exploratory insight into the *CYP21A2* variants among Indonesian CAH individuals. Considering the complexity of gene rearrangements and various SNVs, incorporating direct sequencing in combination with other method, i.e., MLPA, would enable more comprehensive detection of new or rare variants.[8]

CONCLUSION

The study presented the feasibility of PCR-RFLP as an applicable screening method for detecting p.Gln318Ter of the *CYP21A2* gene causing SW CAH in a resource-limited setting. This method was appropriate because of the accessibility of the equipment and materials used with a minimum requirement of PCR and gel electrophoresis. Genotype-phenotype correlation could not be conducted because the p.Gln138Ter was found in heterozygous state and no other targeted variants were found. Nevertheless, a more advanced molecular technique, such as MLPA combined with Sanger sequencing, is recommended due to its ability to discover novel variants and more comprehensive analysis in CAH patients, thereby enabling correct diagnosis, early and personalized treatment, and genetic counseling

ACKNOWLEDGMENTS

The authors would like to express gratitude to the CAH patients for participating in the study and the lab technicians at the Center for Biomedical Research (CEBIOR), Faculty of Medicine Universitas Diponegoro, for assisting in the research process. The study was funded by *Pendidikan Magister menuju Doktor untuk Sarjana Unggul* (PMDSU) scholarship (No. 359-075/UN7.D2.1/PP/VI/2025) by the Ministry of Higher Education, Science, and Technology of the Republic of Indonesia.

REFERENCES

1. Claahsen - van der Grinten HL, Speiser PW, Ahmed SF, et al. Congenital Adrenal Hyperplasia—Current Insights in Pathophysiology, Diagnostics, and Management. *Endocr Rev*. 2022;43(1):91-159. DOI:10.1210/endrev/bnab016
2. Speiser PW, Arlt W, Auchus RJ, et al. Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency: An Endocrine Society* Clinical Practice Guideline. *J Clin Endocrinol Metab*. 2018;103(11):4043-4088. DOI:10.1210/jc.2018-01865
3. Armstrong K, Benedict Yap A, Chan-Cua S, et al. We All Have a Role to Play: Redressing Inequities for Children Living with CAH and Other Chronic Health Conditions of Childhood in Resource-Poor Settings. *Int J Neonatal Screen*. 2020;6(4):76. DOI:10.3390/ijns6040076
4. Adriaansen BPH, Utari A, Westra D, et al. 46,XX males with congenital adrenal hyperplasia: a clinical and biochemical description. *Front Endocrinol*. 2024;15. DOI:10.3389/fendo.2024.1410122
5. Utari A, Faradz SMH, Ediati A, et al. Challenges in the treatment of late-identified untreated congenital adrenal hyperplasia due to CYP11B1 deficiency: Lessons from a developing country. *Front Endocrinol*. 2022;13:1015973. DOI:10.3389/fendo.2022.1015973
6. Yang M, White PC. Genetics and Pathophysiology of Classic Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab*. 2025;110(Supplement_1):S1-s12. DOI:10.1210/clinem/dgae535
7. Maher JY, Gomez-Lobo V, Merke DP. The management of congenital adrenal hyperplasia during preconception, pregnancy, and postpartum. *Rev Endocr Metab Disord*. 2023;24(1):71-83. DOI:10.1007/s11154-022-09770-5
8. Krone N, Arlt W. Genetics of congenital adrenal hyperplasia. *Best Pract Res Clin Endocrinol Metab*. 2009;23(2):181-192. DOI:10.1016/j.beem.2008.10.014
9. Faradz SMH, Listyasari N, Utari A, et al. Lessons Learned from 17 Years of Multidisciplinary Care for Differences of Sex Development Patients at a Single Indonesian Center. *Sex Dev*. Published online September 12, 2023:1-11. DOI:10.1159/000534085
10. Zhang B, Lu L, Lu Z. Molecular diagnosis of Chinese patients with 21-hydroxylase deficiency and analysis of genotype–phenotype correlations. *J Int Med Res*. 2017;45(2):481-492. DOI:10.1177/0300060516685204
11. Yokoyama Y, Teraoka M, Tsuji K, et al. Rapid screening method to detect mutations in CYP21, the gene for 21-hydroxylase. *Am J Med Genet*. 2000;94(1):28-31. DOI: 10.1002/1096-8628(20000904)94:1<28::AID-AJMG6>3.0.CO;2-F
12. Asanuma A, Ohura T, Ogawa E, et al. Molecular analysis of Japanese patients with steroid 21-hydroxylase deficiency. *J Hum Genet*. 1999;44(5):312-317. DOI:10.1007/s100380050167
13. Shvartsman E, Richmond MEI, Schellenberg JJ, et al. Comparative analysis of DNA extraction and PCR product purification methods for

cervicovaginal microbiome analysis using cpn60 microbial profiling. *PloS One*. 2022;17(1):e0262355. DOI:10.1371/journal.pone.0262355
14. Pedersen LMI, Stangegaard M, Mogensen HS, Morling N. Purification and concentration of PCR products leads to increased signal intensities with fewer allelic drop-outs and artifacts. *Prog Forensic Genet 14*. 2011;3(1):e347-e348. DOI:10.1016/j.fsigss.2011.09.036
15. Smith PJ, Ballantyne J. Simplified Low-Copy-Number DNA Analysis by Post-PCR Purification. *J Forensic Sci*. 2007;52(4):820-829. DOI:10.1111/j.1556-4029.2007.00470.x
16. Hannah-Shmouni F, Chen W, Merke DP. Genetics of Congenital Adrenal Hyperplasia. *Endocrinol Metab Clin*. 2017;46(2):435-458. DOI:10.1016/j.ecl.2017.01.008
17. Espinosa Reyes TM, Collazo Mesa T, Lantigua Cruz PA, Agramonte Machado A, Domínguez Alonso E, Falhammar H. Genotype-Phenotype Correlation in Patients with Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency in Cuba. *Int J Endocrinol*. 2021;2021:9316284. DOI:10.1155/2021/9316284
18. Liu Y, Zheng J, Liu N, et al. The spectrum of CYP21A2 gene mutations in patients with classic salt wasting form of 2l-hydroxylase deficiency in a Chinese cohort. *Mol Genet Genomic Med*. 2020;8(11):e1501. DOI:10.1002/mgg3.1501
19. Wang X, Wang Y, Ma D, et al. Neonatal Screening and Genotype-Phenotype Correlation of 21-Hydroxylase Deficiency in the Chinese Population. *Front Genet*. 2020;11:623125. DOI:10.3389/fgene.2020.623125
20. Khajuria R, Walia R, Bhansali A, Prasad R. The spectrum of CYP21A2 mutations in Congenital Adrenal Hyperplasia in an Indian cohort. *Clin Chim Acta*. 2017;464:189-194. DOI:10.1016/j.cca.2016.11.037
21. Riedl S, Röhl FW, Bonfig W, et al. Genotype/phenotype correlations in 538 congenital adrenal hyperplasia patients from Germany and Austria: discordances in milder genotypes and in screened versus prescreening patients. *Endocr Connect*. 2019;8(2):86-94. DOI:10.1530/EC-18-0281
22. Stikkelbroeck NMML, Hoefsloot LH, de Wijs IJ, Otten BJ, Hermus ARMM, Sistermans EA. CYP21 Gene Mutation Analysis in 198 Patients with 21-Hydroxylase Deficiency in The Netherlands: Six Novel Mutations and a Specific Cluster of Four Mutations. *J Clin Endocrinol Metab*. 2003;88(8):3852-3859. DOI:10.1210/jc.2002-021681
23. Kurzyńska A, Skalniak A, Franson K, Bistika V, Hubalewska-Dydejczyk A, Przybylik-Mazurek E. Molecular analysis and genotype-phenotype correlations in patients with classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency from southern Poland — experience of a clinical center. *Hormones*. 2022;21(2):241-249. DOI:10.1007/s42000-022-00348-z
24. Fardella CE, Poggi H, Pineda P, et al. Salt-Wasting Congenital Adrenal Hyperplasia: Detection of Mutations in CYP21B Gene in a Chilean Population1. *J Clin Endocrinol Metab*. 1998;83(9):3357-3360. DOI:10.1210/jcem.83.9.5071
25. Arriba M, Ezquieta B. *Molecular Diagnosis of Steroid 21-Hydroxylase Deficiency: A Practical Approach*. Vol 13.; 2022. DOI:10.3389/fendo.2022.834549
26. Radu M, Moldovan R, Băban A. Families with complex needs: an inside perspective from young people, their carers, and healthcare providers. *J Community Genet*. 2022;13(3):293-302. DOI:10.1007/s12687-022-00586-z
27. Higgs E, Wain KE, Wynn J, et al. Measuring quality and value in genetic counseling: The current landscape and future directions. *J Genet Couns*. 2023;32(2):315-324. DOI:10.1002/jgc4.1657
28. Madlensky L, Trepanier AM, Cragun D, Lerner B, Shannon KM, Zierhut H. A Rapid Systematic Review of Outcomes Studies in Genetic Counseling. *J Genet Couns*. 2017;26(3):361-378. DOI:10.1007/s10897-017-0067-x
29. Hashim HO, Al-Shuhaib MBS. Exploring the Potential and Limitations of PCR-RFLP and PCR-SSCP for SNP Detection: A Review. *J Appl Biotechnol Rep*. 2019;6(4):137-144. DOI:10.29252/JABR.06.04.02
30. Chial H. DNA fingerprinting using amplified fragment length polymorphisms (AFLP): No genome sequence required. Nature Education. 2008. Accessed June 26, 2024. https://www.nature.com/scitable/topicpage/dna-fingerprinting-using-amplified-fragment-length-polymorphisms-39051/
31. Yang W, Kang X, Yang Q, Lin Y, Fang M. Review on the development of genotyping methods for assessing farm animal diversity. *J Anim Sci Biotechnol*. 2013;4(1):2. DOI:10.1186/2049-1891-4-2
32. Sakari SL, Jimson S, Masthan KMK, Jacobina J. Role of DNA profiling in forensic odontology. *J Pharm Bioallied Sci*. 2015;7(Suppl 1):S138-141. DOI:10.4103/0975-7406.155863
33. Neocleous V, Fanis P, Phylactou LA, Skordis N. *Genotype Is Associated to the Degree of Virilization in Patients With Classic Congenital Adrenal Hyperplasia*. Vol 9.; 2018. DOI:10.3389/fendo.2018.00733
34. Fu X, Shi Y, Ma J, et al. Advances of Multiplex Ligation-dependent Probe Amplification Technology in Molecular Diagnostics. *BioTechniques*. 2022;73(4):205-213. DOI:10.2144/btn-2022-0017
35. Yuan D, Cai R, Mao A, et al. Improved genetic characterization of congenital adrenal hyperplasia by long-read sequencing compared with multiplex ligation-dependent probe amplification plus Sanger sequencing. *J Mol Diagn*. Published online June 24, 2024. DOI:10.1016/j.jmoldx.2024.05.009