

<https://doi.org/10.33472/AFJBS.6.1.2024.20-29>

African Journal of Biological Sciences

Journal homepage: <http://www.afjbs.com>

Research Paper

Open Access

Molecular Detection of Five Types of Thalassemia Mutations among Patients with B-Thalassemia in Northern Basrah Regions Using ARMS-PCR Technology

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Article Info

Volume 6, Issue 1, January 2024

Received: 17 November 2023

Accepted: 08 December 2023

Published: 05 January 2024

doi: [10.33472/AFJBS.6.1.2024.20-29](https://doi.org/10.33472/AFJBS.6.1.2024.20-29)

ABSTRACT

Background: ARMS-PCR technology has been employed for the examination of genetic mutations associated with hematological disorders. In all instances where ARMS-PCR reactions were deemed successful, an output of 861 base pairs was identified, serving as a reliable indicator of reaction success during gel electrophoresis. The ARMS-PCR products were closely monitored and analyzed under both normal and mutated conditions. The product lengths exhibited variability depending on the specific mutation type and its location. For the IVS-I-5(G-to-C) mutation, the ARMS-PCR assay yielded a 285-base pair output, confirming the presence of this mutation in one male thalassemia sample. In the case of Codon 8/9 (+ G), the ARMS-PCR generated a 225-base pair output, and this mutation was identified in two male thalassemia samples. For the Codon 41/42 mutation (-TTCT), an ARMS-PCR output of 439 base pairs was observed, and this mutation was detected in one male thalassemia sample. As for the Codon 30(G-to-C) mutation, no ARMS-PCR output was detected, indicating its absence in the studied samples. Finally, the Codon 8 mutation (-AA) produced an ARMS-PCR output of 255 base pairs. Although this mutation was observed in the study, it was not detected in the samples under investigation.

Aim: The study aims to diagnose five types of mutations that cause beta-thalassemia in the northern areas of Basra.

Material and Methods: Blood samples were obtained from intravenous injections of patients using a single-use syringe (5 ml) placed in EDTA tubes that prevent blood clots and kept in deep freeze (-20oC) until use in genetic detection. The genomic DNA was isolated from the blood completely using an extraction kit and is done according to the manufacturer's instructions (Geneaid, USA).

Results: The three mutations (IVS-I-5(G-to-C), Codon 8/9 (+ G), and Codon 41/42) were found in the studied samples, while the two mutations (Codon 8(-AA) and Codon 30(G-to-C)) were not found in the studied samples.

Conclusion: This study has provided valuable insights into the presence of genetic mutations responsible for blood disorders in the examined samples. These findings contribute significantly to our comprehension of the etiology of these diseases and the development of diagnostic and therapeutic approaches.

Keywords: Mutation, β -thalassemia, ARMS-PCR, Basrah

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1. Introduction

Thalassemia encompasses a group of conditions where the structure of hemoglobin remains normal but its quantity varies (Jord et al., 2000). This is an autosomal genetic disorder that can be inherited from parents without regard to gender (Davies, 2000).

There are two primary types of thalassemia: alpha thalassemia and β -thalassemia, contingent on which globin is mutated. Alpha thalassemia arises when one or more of the four alpha-globin genes are impaired, while β -thalassemia occurs when both β -globin genes sustain damage or mutations (Kim and Tridane, 2017). This mutation affects the production of hemoglobin, resulting in quantitative abnormalities, distinguishing it from hemoglobinopathies, which pertain to specific hemoglobin disorders (Mohan, 2000).

Thalassemia is a common genetic disorders, posing both physical and psychological challenges that significantly impact the quality of life, particularly in regions like the Mediterranean and Southeast Asia where 5% of individuals carrying the thalassemia gene are affected (Piel and Weatherall, 2014).

Alpha thalassemia (α) and β -thalassemia (β) are the two primary forms of thalassemia, with alpha-thalassemia occurring when one or more of the four α -globin genes are defective and β -thalassemia manifesting when all β -globin genes are mutated (Kim and Tridane, 2017).

Major thalassemia results from inheriting two faulty globin genes, one from each parent, while minor thalassemia stems from inheriting only one defective globin gene from one parent (Cao and Galanello, 2010; Danjou et al., 2011). Thalassemia minor exhibits no clinical symptoms, allowing individuals to lead healthy lives without treatment. Conversely, thalassemia major leads to lifelong anemia starting in early childhood, necessitating regular blood transfusions due to irregularities in red blood cell production (Kim & Tridane, 2017).

Alpha homozygous thalassemia results from the deletion of all four α -globin genes, while β -thalassemia displays varying phenotypes, ranging from severe blood-transfusion-dependent cases to milder intermediate forms. Individuals with β -thalassemia major experience severe anemia, enlarged liver and spleen, and if left untreated, can suffer from mental retardation and reduced life expectancy (Danjou et al., 2011).

The prevalence of hemoglobin disorders in northern Basra is influenced by several factors, including genetic diversity among different ethnic groups residing in the region, such as Arabs, Kurds, and Iranians. These genetic variations can contribute to hemoglobin disorders. Research by Al-Ali et al. (2012) discovered a higher incidence of the sickle cell gene among people in northern Basra compared to other parts of Iraq, indicating a genetic basis for the elevated occurrence of sickle cell anemia in this region.

Furthermore, the distribution of hemoglobin disorders can be affected by genetic diversity within populations and the prevalence of specific genetic mutations (Williams and Weatherall, 2012). The frequency of particular hemoglobin disorders, such as β -thalassemia or sickle cell disease, may vary across different regions depending on the prevalence of the responsible gene mutations (Hassan et al., 2003), as observed in variations in the distribution of β -thalassemia and hemoglobin S (sickle cell trait) across different areas of Basrah (Risoluti et al., 2021).

2. Methodology

The study focused on examining a cohort of 24 individuals under the care of the Center for Genetic Hematology and the Hematology Laboratory, with meticulous record-keeping and consistent monitoring. Disease evaluation was conducted using a high-performance liquid chromatography (HPLC) system obtained from Bio-Rad Laboratories in Hercules, CA, USA, specifically employing the Variant β -Short program.

Intravenous injections were administered to the patients using a disposable 5 ml syringe for blood sample collection, which was then transferred into EDTA tubes to prevent coagulation. These tubes were preserved at -20°C until needed for genetic assessment. The genomic DNA was extracted entirely from the blood samples using a DNA extraction kit, in adherence to the manufacturer's guidelines, supplied by Geneaid in the USA.

• Primers Selection

1. A series of prefixes were employed in the analysis of mutations using the amplification-refractory mutation system (ARMS), as outlined in Table 1 (Talmaci et al., 2004; Baig et al., 2005; Basak, 2007; Mirasena et al., 2008; Sarookhani et al., 2009; Saleh-Gohari et al., 2010).
2. The oligonucleotides corresponding to the chosen prefixes utilized in the ARMS technology are provided below. These primers were employed as internal control primers for all ARMS-PCR reactions:
 - “Forward Primer: 5-CAA TGT ATC ATG CCT CTT TGC ACC -3”
 - “Reverse Primer: 5-GAG TCA AGG CTG AGA GAT GCA GGA -3”
 - “Common Primer C: 5-ACC TCA CCC TGT GGA GCC AC -3”
 - “Common Primer D:5-CCC CTT CCT ATG ACA TGA ACT TAA-3

Table (1) Sequence of prefixes and sizes of their products for mutations identified in the study

Mutation	Sequence(5'-3')	Common primer	Product (bp)
IVS- I- 5 (G – C)	5-CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG-3	C	285
Normal	5-CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC-3		
Codon 8 \ 9 (+ G)	5-CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC-3	C	225
Normal	5-CCT TGC CCC ACA GGG CAG TAA CGG CAC ACT-3		
Codon 8 (-AA)	5- ACA CCA TGG TGC ACC TGA CTC CTG AGC ACG3	D	520
Normal	5-ACA CCA TGG TGC ACC TGA CTC CTG AGC AGA-3		
Codon 41/42(-TCTT)	5-GAG TGG ACA GAT CCC CAA AGG ACT CAA CCT-3	C	439
Normal	5-GAGTGG ACA GAT CCC CAA AGG ACT CAA AGA-3		
Codon 30 (G-C)	5-TAA ACC TGT CTT GTA ACC TTG ATA CCT ACG-3	C	280
Normal	5-TAA ACC TGT CTT GTA ACC TTG ATA CCT ACC- 3		

• ARMS-PCR Programmers

A final volume of 20 µl was utilized for the PCR reaction, which consisted of 5 µl of master mix (provided by Bioneer in Korea), 0.5 µg of DNA template, and 10 pmol of each primer. The primers included 2 internal control primers, 1 common primer, and 1 ARMS primer for either the mutant or normal reaction. The thermal cycling conditions for IVS1-5, codon 30,

codon 8/9, and Cd41/42 involved 30 cycles. This encompassed denaturation at 94°C for 1 minute, primer annealing at 65°C for 1 minute, and extension at 72°C for 90 seconds. This was followed by a final extension step at 72°C for 3 minutes. For codon 8 mutations, the same protocol was applied, except for the annealing temperature, which was set to 61°C. Subsequently, the PCR products underwent analysis through 2% agarose gel electrophoresis.

3. Results and Discussion

In this research, the ARMS-PCR method was employed to perform molecular screening and identify seven distinct β -thalassemia mutations. Each mutation was targeted using a unique set of primers, alongside a set of internal control primers, as part of the detection process.

- Molecular detection of β -thalassemia mutations:

In all the ARMS-PCR reactions that yielded successful results, we observed an internal control product with a molecular weight of 861 base pairs. This product served as a crucial indicator for the success of the reaction during gel electrophoresis. Its size fell within the range of 800 to 900 base pairs, as guided by a 100 base pair ladder marker. Additionally, we detected ARMS-PCR products for individuals in normal, heterozygous, and/or homozygous states for each studied mutation.

For individuals in a normal state, ARMS-PCR products were exclusively found in the reactions with normal primers. In contrast, heterozygous cases exhibited ARMS-PCR products in both normal and mutant reactions. Homozygous states, on the other hand, displayed ARMS-PCR products solely within the reactions containing mutant primers.

- The product of the studied species of β -thalassemia mutations were presented as follows:

A – In figure (1), an ARMS-PCR result of 285 base pairs was detected for the IVS-I-5(G-to-C) mutation. The IVS-I-5 mutation's ARMS-PCR output was visualized on a 1% agarose gel. In each sample, an internal control package of 861 base pairs was present, and the first lane represents the size markers of the DNA ladder (100 base pairs).

Sample (1) exhibited an amplified product with the normal primer (N) and no amplification with the mutant primer (M), indicating the presence of a healthy individual. In contrast, Sample (6) showed amplification solely with the mutant primer (M), signifying a homozygous genetic type.

Our study revealed a mutated product of 285 base pairs in Sample (6) of male thalassemia patients, with a frequency of 4.17% (Figure 1). This finding indicates the existence of a β -thalassemia IVS-1-5 (G-to-C) mutation within this sample. This discovery contributes to our understanding of the factors contributing to thalassemia in the studied region, improving early diagnosis and suitable treatment. It is important to note that our study identified the IVS-1-5 (G-to-C) mutation in only one male patient, underscoring the necessity for further investigations with larger patient cohorts to determine the mutation's prevalence in the region and its association with symptom development and severity in thalassemia cases.

Our current study identified a 285 bp mutated product in Sample (6) of male thalassemia patients, occurring at a frequency of 4.17% with a homozygous genotype (Fig. 1). This result aligns with previous research conducted in both the southern and northern regions of Basra Governorate (Al-Badran et al., 2016).

Iraq has a relatively high prevalence of thalassemia, including β -thalassemia, with various thalassemia mutations identified in the Iraqi population in previous studies (Al-Allawi et al., 2013; Al-Allawi and Jubrael, 2014; Musa et al., 2017).

Furthermore, the IVS-1-5 (G-to-C) mutation has been noted as a recurrent mutation in southern Iran (Najmabadi et al., 2001), Balochistan and Sindh provinces in Pakistan (Khan and Riazuddin, 1998), and neighboring countries such as the western region of Saudi Arabia, Bahrain, Kuwait, United Arab Emirates, and Oman, with prevalence ranging from 16.2% to

66.2% (Al-Ali et al., 2005; Saleh-Gohari and Bazrafshani, 2010). This data suggests that IVSI-5 is a dominant mutation in the countries surrounding the Persian Gulf and southern Iran, possibly influenced by gene flow in the region.

B- In figure (1), we observed an ARMS-PCR output of 255 base pairs for the Codon 8/9 (+G) mutation. The ARMS-PCR output for this Codon 8/9 mutation was analyzed using a 1% agarose gel. Each sample included an internal control package with a size of 861 base pairs, and the first lane depicted the DNA ladder marker (100 base pairs).

Samples (2) and (3) both displayed an amplification product with both natural (N) and mutant (M) primers, indicating a heterozygous genotype. In contrast, Sample (1) showed an amplification product with the natural primer (N) but not with the mutant primer (M), signifying the presence of a healthy individual.

Our study revealed the presence of the Codon 8/9 (G+) mutation in samples 2 and 3 of male thalassemia patients (Figure 1) at a frequency of 8.34%. This confirms the existence of this genetic mutation in these samples, based on the size of the electrophoresed fragment (255 base pairs). It indicates that the DNA in these two samples carries the Codon 8/9 (+G) β -thalassemia mutation. This discovery contributes significantly to our understanding of the underlying factors for thalassemia in this geographic area, potentially aiding in the development of early diagnostic tests and appropriate preventive and therapeutic measures for affected patients.

The Codon 8/9 (+G) mutation is a recurrent mutation associated with β -thalassemia in individuals with hemoglobinopathy in Iraq, particularly in cases of major β -thalassemia (Al-Allawi et al., 2010; Al-Badran et al., 2016). This mutation is also referred to as the Indo-Asian mutation or β^0 Asian Indian mutation (Al-Badran et al., 2016). The distribution of the Codon 8/9 (+G) mutation appears to vary across different regions of Iraq, with a higher prevalence noted in central and northern Iraq (Auda et al., 2019). It is essential to highlight that this mutation is among the seven most common mutations found in a significant proportion of β -thalassemia chromosomes among Iraqi patients (Al-Allawi et al., 2010). The elevated incidence of this mutation in the population contributes to its prevalence among individuals with hemoglobin disorders in Iraq.

The presence of the Codon 8/9 (+G) mutation is not limited to Iraq but has also been reported in other populations globally. This underscores the genetic diversity and complexity of β -thalassemia worldwide. Al-Abboodi (2011) reported a frequency rate of 4.1% for the Codon 8/9 (+G) mutation in Baghdad. Additionally, the frequency rate of this mutation among thalassemia patients in Iraq (Baghdad) was 39.5% (Saud, 2012).

Several studies have offered explanations for the presence of the Codon 8/9 (+G) gene mutation in patients with hemoglobin disorders in Iraq and around the world. Al-Allawi et al. (2010) noted that the Codon 8/9(+G) mutation accounted for approximately 15% of β -thalassemia cases in Iraq and suggested that its high frequency might be due to historical migration patterns or inherent effects.

Studies conducted in neighboring countries have also indicated the presence of the Codon 8/9 (+G) mutation, suggesting a shared genetic heritage. Weatherall et al. (2001) reported the widespread occurrence of this mutation in various populations worldwide, including Mediterranean countries, India, Iran, and Iraq. They proposed that this mutation may have originated in the Mediterranean region and subsequently spread globally through migration.

Kountouris et al. (2014) suggested that the presence of the Codon 8/9 (+G) mutation in Iraq could be attributed to historical interactions between Iraq and other Mediterranean populations. Furthermore, the high prevalence of this mutation in Iraq may be linked to the practice of consanguineous marriages, which increase the likelihood of inherited genetic disorders (Hamamy and Al-Allawi, 2013). β -thalassemia is notably prevalent in the Middle East, and the Codon 8/9 (+G) mutation has been reported in other countries within the region,

emphasizing the role of multiple factors in its presence among Iraqi patients with hemoglobinopathy.

The Codon 8/9 (+G) mutation is also prevalent in the Indian subcontinent and northwestern Iran, with frequency rates of 14.5% and 15.7%, respectively. Its distribution appears to decrease as one moves westward and northward towards Dohuk Province in Iraq and the Eastern Anatolia region of Turkey (Basak, 2007; Pehlivan et al., 2010).

C - In figure (1), we observed an ARMS-PCR output of 439 base pairs for the Codon 41/42 (-TTCT) mutation. The ARMS-PCR output for this Codon 41/42 mutation was visualized on a 1% agarose gel. All samples included an internal control package with a size of 861 base pairs, and the first lane represented the DNA ladder marker (100 base pairs). Sample (9) exhibited an amplification product with both the natural (N) and mutant (M) primers, indicating a heterozygous genotype for this individual.

The findings from Figure 1 in our study revealed the presence of a mutated fragment measuring 439 base pairs in Sample No. 7 of male thalassemia patients, occurring at a frequency of 4.17%. This observation indicates the existence of a β -thalassemia mutation of the Codon 41/42 (-TTCT) type in this sample. This discovery holds significant importance in our efforts to comprehend the underlying factors contributing to thalassemia in the studied region, pinpointing the genetic mutations responsible for the disease. Additionally, it contributes to the enhancement of early diagnostic capabilities for affected patients, enabling the implementation of appropriate treatment measures.

It is noteworthy that our study identified the Codon 41/42 (-TTCT) mutation in only one male patient among those examined. Therefore, it is advisable to conduct further extensive research and sample analysis to determine the prevalence of this mutation within the region and confirm its association with the disease. It's worth mentioning that our findings differ from those reported by Saud (2012), who did not document the presence of the mutation in question in Baghdad governorate.

D - In figure (1), we observed an ARMS-PCR output of 280 base pairs for the Codon 30(G-to-C) mutation. The ARMS-PCR output for this Codon 30 mutation was displayed on a 1% agarose gel. All samples were equipped with an internal control fragment (861 base pairs), and the first lane represented the DNA ladder marker (100 base pairs).

As per our study, no mutated fragments with a length of 280 base pairs were detected, indicating the absence of a Codon 30 (G-to-C) mutation associated with β -thalassemia in the analyzed samples. This result underscores the significance of utilizing electrophoresis gel technology to identify and confirm genetic mutations responsible for β -thalassemia. It also contributes to the advancement of early diagnostic capabilities and appropriate preventive measures. When conducting genetic diagnoses of thalassemia in the northern region of Basra Governorate, it's essential to take into consideration the absence of the Codon 30 (G-to-C) mutation in these samples.

However, it's important to note that the absence of the Codon 30 (G-to-C) mutation in these samples does not imply the absence of other mutations that may cause thalassemia. It is advisable to conduct further research and analysis on larger sample sets to determine the prevalence of various genetic mutations responsible for thalassemia in the region. This finding aligns with Saud's (2012) report, which also did not document the presence of the mutation under investigation in Baghdad governorate.

There could be several potential reasons for the lack of recorded instances of the Codon 30 (G-to-C) mutation associated with β -thalassemia in patients with hemoglobinopathy in Iraq. One plausible explanation could be that this mutation is less common in the Iraqi population compared to other populations where it has been identified. Additionally, the absence of comprehensive genetic studies and screening programs specifically targeting this mutation in Iraq may contribute to its apparent absence in documented cases of

hemoglobinopathy (Saeed, 2012).

E - In figure (1), we observed an ARMS-PCR output of 255 base pairs for the Codon 8(-AA) mutation. The ARMS-PCR output for this Codon 8 mutation was visualized on a 1% agarose gel. All samples were equipped with an internal control fragment (861 base pairs), and the first lane represented the DNA ladder marker (100 bp). The findings presented in Figure 5 indicate that no mutated fragments of 520 base pairs were observed, signifying the absence of a Codon 8(-AA) β -thalassemia mutation in the samples under investigation. This result reinforces the notion that this mutation has not spread within the studied area and underscores the significance of employing advanced molecular techniques for the identification and screening of genetic mutations responsible for thalassemia. However, it's important to note that this finding may pertain solely to the samples examined in this study. Therefore, it is advisable to conduct further extensive research and sample analysis on a larger scale to determine the prevalence of this mutation in the region and across different genders while exploring its association with the disease.

Our study's result aligns with Saud's (2012) study, which demonstrated a higher presence of this mutation in northern Iraq. Codon 8 (-AA) is a common Mediterranean mutation in the Eastern Mediterranean region (Zahed, 2001; Al-Allawi et al., 2006). Moreover, the majority of chromosomes carrying this mutation were reported in Erbil, suggesting regional variations even within the same ethnic group (Saud, 2012).

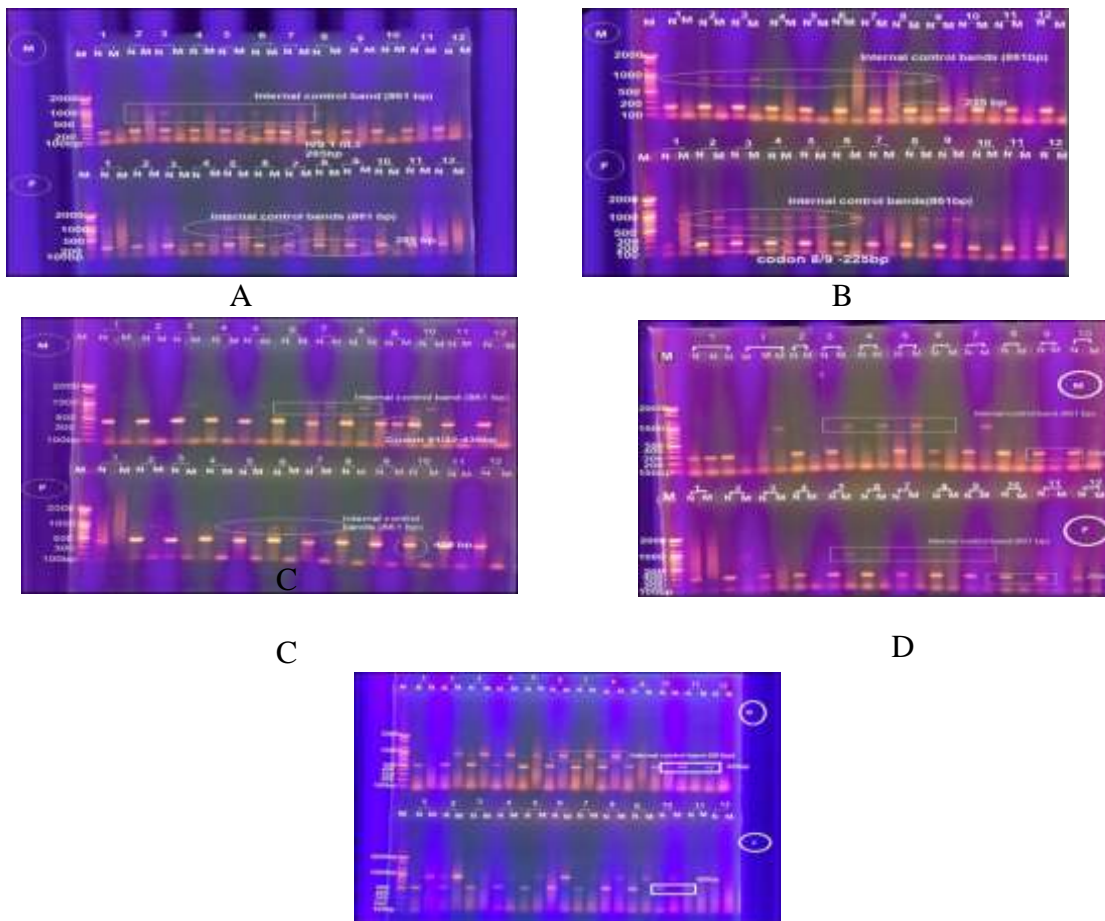


Figure (1) ARMS-PCR products on 2% agarose gel electrophoresis at 60 voltages for 45 min. A-IVS-1-5(G-C) mutation (285 bp); B-Codon 8/9 (+G) mutation (225 bp); C-Codon 41/42 (-TCTT) mutation (439 bp); D-Codon 30 (G-C) mutation (280 bp); E-Codon 8 (+AA) mutation

(255 bp); Marker: ladder marker (100 bp) DNA; N: normal; M: mutant.

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Cite this article as: Eman Salah Jemaa (2024).

Molecular Detection of Five Types of Thalassemia Mutations among Patients with B-Thalassemia in Northern Basrah Regions Using ARMS-PCR Technology

African Journal of Biological Sciences. 6(1), 20-29. *doi: 10.33472/AFJBS.6.1.2024.20-29*