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BREEDING FOR ENHANCED YIELD AND QUALITY TRAITS IN COWPEA (*Vigna unguiculata* L.)

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Cowpea (*Vigna unguiculata* L. Walp.; 2n =2x = 22) is a pivotal crop cultivated extensively in low-in put production systems and arid and semi-arid agro-ecologies globally (Boukar *et al*., 2019). As a legume within the family Fabacea and sub-family Faboideae (Padulosi and Ng, 1997 and Agbogidi, 2010), cowpea, characterized by low outcrossing and high self-pollination, serves as a valuable source of low-cost protein (17 to 25%) with essential amino acids, lysine and tryptophan (Rangel *et al.*, 2003; Ibro *et al.*, 2014). Recognized as the "poor man's

meat" in many developing countries (Jayathilake *et al.,* 2018), cowpea thrives in challenging environmental conditions, contributing to soil fertility through nitrogen fixation in crop rotation (Bado *et al.,* 2006; Dugje *et al.,* 2009 and Gnanamurthy *et al*., 2012).

Driven by rapid population growth in Egypt, recent research focuses on enhancing cowpea yield quantity and quality through intensive breeding efforts, reliant on the presence of genetic variability enabling effective selection. The selection of superior genotypes correlates with the extent of genetic variability and the heritability of the inherited characteristics (Scarano *et al*., 2014). Understanding the magnitude and type of genetic variability, along with corresponding heritability, is crucial in breeding programs for improving crop yield and quality traits. Therefore, investigating the relationship between genotype variability and yield components is essential for the efficient utilization of cowpea genetic resources in the context of Egyptian agricultural productivity.

In most crop improvement programs, enhancing yield stands as a primary breeding objective (More and Borkar, 2016). Cowpea yield, being a quantitative trait, is intricately linked to numerous morphological, physiological and agronomic traits, influenced by both genetic and environmental factors. The efficacy of selection relies on the availability of substantial genetic variability within the breeding material for the target character and its heritability (Atta *et al.,* 2008). The direction and magnitude of associations between traits to be improved also play a crucial role (More and Borkar, 2016). Thus, studying the genetic variability and heritability of yield and its associated traits is paramount for yield improvement. Plant genetic resources exhibit variation that supports the selection of superior genotypes and the development of improved cultivars with desirable characteristics.

Protein markers and DNA markers can be used for assessment genetic variability based on morphological traits which influenced by environmental factors (El-Shazly *et al.,* 2020). Retrotransposons are ubiquitous and abundant transposable elements in eukaryotic genomes which classified into long terminal repeats (LTRs) and non-LTRs (Kumar and Bennetzen, 1999). Retrotransposons are dispersed throughout plant genomes and some retrotransposon families are represented by thousands of copies (Kalendar *et al.,* 2010). New copies of retrotransposons are randomly inserted into preexisting sequences of the genome *via* a copy-paste system, which consequently increases the copy number (Kalendar and Schulman, 2007). Retrotransposons contribute to the size, structure, variation, and diversity of the genome. In addition, they greatly effect gene function and cover a high percentage of the genome (Gbadegesin and Beeching, 2010).

They are known to insert themselves into the genome and act as muta-

genic agents thereby providing a potential source of gene diversity (Bourque *et al.*, 2018). Among the transposable element based markers, new retrotransposon-based DNA fingerprinting techniques, IRAP (Inter Retrotransposon Amplified Polymorphism) that produce dominant, multiplex marker systems that examine variation in retrotransposon insertion sites. IRAP makes use of conserved retrotransposon sequences termed LTRs for detection of polymorphism. It is based on the amplification of regions between two neighboring retrotransposon.

IRAPs serve as effective molecular markers owing to the abundance of retrotransposon copies in plant genomes and their ability to generate new copies (Kalendar and Schulman, 2013). RTN markers possess advantages of easy assessment, low cost, and high in formativeness and polymorphism (Bhandari *et al*., 2017). Consequently, IRAP markers provide an efficient DNA fingerprint for each genotype, enabling genetic identification and kinship assessment (Badr, 2008). The effectiveness of IRAP analysis has been demonstrated in various studies, such as those on *Medicago sativa* L. landraces and Iranian bread wheat cultivars and breeding lines (Annicchiarico, 2006; Nasri *et al*., 2013; Farouji *et al*., 2015 and Taheri *et al*., 2018). Additionally, IRAP has been applied in phylogenetic analyses among commercial triploids and their wild relatives in Musa germplasm (Somasundaram *et al*., 2023) and for fingerprinting, diversity studies, and linkage maps in yeast and barley (Shehata *et al*., 2015).

Despite extensive literature, there is a dearth of reports on the use of IRAP markers to assess the genetic diversity of cowpea (*Vigna unguiculata* L.) genotypes in Egypt. This study aims to fill this gap by providing insights into the genetic diversity of the country's cowpea germplasm using IRAP markers. Additionally, the research evaluates genetic variability and heritability among cowpea genotypes for yield and related traits, aiming to identify promising lines with maximum productivity and high seed quality under Egyptian conditions.

MATERIALS AND METHODS

This research was conducted over the span of 2022 to 2023 under open field conditions at Qaha Vegetable Research Farm, Horticultural Research Institute (HRI), Agriculture Research Center (ARC), situated in Qalyoubia Governorate, Egypt. A comprehensive selection of 20 breeder-chosen lines of cowpea (*Vigna unguiculata* L. Walp) and five commercially established cultivars (Balady, Cream 7, Kafr Elsheikh 1, Qaha 1, and Tiba) were utilized in this study. All entries were sourced from the Horticultural Research Institute, Agriculture Research Center (ARC), Egypt. The identification of promising lines was based on criteria such as earliness, seed quality, and high seed yield, as illustrated in Table (1) and Fig. (1). It is noteworthy that there was observed variation in seed color among these entries.

The evaluation took place over two consecutive summer seasons in 2022 and

2023, with combined data across both seasons being calculated. The seeds of the twenty-five genotypes (comprising twenty selected lines and five commercial cultivars) were sown in the first week of May during both seasons. A randomized complete block design with three replicates was employed, with each plot consisting of three rows. The seeds were sown on raised beds, maintaining an 80 cm row-to-row spacing and a 15 cm plant-to-plant spacing at a depth of 5 cm. Standard cultural practices, including irrigation, chemical fertilization, and disease and pest control, were applied in accordance with local practices. Data were systematically collected and recorded on a plot basis, with the mean of each genotype utilized in subsequent statistical analyses. The parameters studied encompassed the number of days to flowering, pod length (cm), number of seeds per pod, 100-seed weight (g), and seed yield (ton/feddan; where one feddan equals 4200 m^2).

Genomic DNA extraction, purification and quantification of 25th cowpea genotypes

Due to the high protein content in cowpea varieties, high molecular weight genomic DNA was isolated from fresh leaves of 25th cowpea genotypes using DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) with some minor modifications by adding PVP (poly venial pyrolidine) to help eliminate phenols, dyes and part of proteins. The quantity and purity of extracted DNA were assessed spectrophotometrically using the ND-1000 system (Nano-Drop Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the Molecular Cloning Laboratory Manual (Maniatis *et al.*, 1988).

IRAP primers - PCR analysis

The IRAP assay, following the methodology outlined by Badr *et al*. (2020), was employed to assess genetic variation within and among 25 cowpea genotypes, utilizing a set of 10 primers (refer to Table 2). The IRAP PCR amplification reactions were conducted in uniform 20µl volumes, comprising 10µl of 2xMaster Mix (One PCRTM, GeneDireX, Inc., Taipei, Taiwan), 2µl of DNA template (15 ng/µl), 2.5µl of primer (10 pc/mol/µl), and 5.5µl of dH_2O.

Amplification reactions were carried out using a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems), with the programmed conditions detailed in Table (3). The resulting amplification products were separated through electrophoresis in a 3% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 120 volts. Visualization of PCR products was achieved under UV light, and images were captured using a Gel Documentation System (BIO-RAD 2000).

Statistical analysis

The acquired data underwent statistical analysis within each season, and subsequently, a combined analysis was performed after confirming the homogeneity of seasons using the method outlined by

Gomez and Gomez (1984). Mean comparisons were conducted using Duncan's multiple range test (Duncan, 1955). The coefficient of variance was computed following the procedure outlined by Steel and Torrie (1981). Genotypic and phenotypic coefficients of variation were estimated based on Burton's methodology (1952). Broad-sense heritability was determined in accordance with the approach proposed by Singh and Chaudhary (1995). The heritability percentage was categorized into low (0-30%), moderate (30–60%), and high (≥60%), following the classification by Johnson *et al.* (1955).

RESULTS AND DISCUSSION

Performance of the selected lines

Table (4) presents a comprehensive overview of the performance of the studied cowpea genotypes for the traits number of days to flowering, pod length, and number of seeds per pod during the 2022 and 2023 seasons, as well as a combined analysis across both seasons. The results reveal substantial variation among the genotypes for each trait.

For the number of days to flowering, considerable diversity was observed, with recorded values ranging from 48.33 days (Line CP 23-1) to 58.83 days (Line CP 66 and cultivar Kafr Elsheikh 1). The overall mean of the selected lines was 53.01 days, while the check cultivars exhibited an overall mean of 55.03 days. This disparity suggests the effectiveness of the selection process in improving the trait.

In the case of pod length, significant differences were noted among the genotypes. CP 25-3 displayed the longest pods (18.04 cm), followed by Kafr Elsheikh 1 and Cream 7 cvs. (17.63 and 17.52 cm, respectively). Notably, CP 23 and CP 25-2 exhibited the shortest pods (12.94 cm and 13.87 cm, respectively), emphasizing the distinctiveness among the studied genotypes.

The number of seeds per pod also exhibited significant differences among the genotypes. Kafr Elsheikh 1 demonstrated the highest number of seeds per pod (13.47), followed by the line CP 25-3 (12.47) without a significant difference between them. These findings emphasize the effectiveness of the selection process in enhancing the seeds per pod trait.

Moving on to the 100-seed weight trait (Table 5), significant differences were evident among the genotypes, with the mean weight ranging from 11.83 g to 18.01 g. The line CP 67 showcased the heaviest seeds (18.01 g), while the line CP 57 exhibited the lowest value (11.83 g). These variations underscore the diverse seed weights among the cowpea genotypes.

The final trait, seed yield per feddan, also displayed significant differences among the genotypes. The selected line CP 65 demonstrated the highest seed yield per feddan (1.456 ton), followed by Tiba (1.431 ton). This suggests the efficacy of the selection process in enhancing seed

yield, with substantial differences observed among the check cultivars.

These results align with previous studies by Ahmed *et al.* (2005), Hussein and Abd El-Hady (2015), and Adams *et al.* (2017), which identified significant differences among cowpea genotypes for traits such as days to 50% flowering, pod length, number of seeds per pod, 100-seed weight, and seed yield. Additionally, Gomes *et al.* (2021) emphasized the high morphological diversity in local landraces, while Boukar *et al.* (2019) attributed the narrow genetic diversity in cowpea to its self-pollinating nature and limited gene flow between wild and cultivated types. Similarly, Lopes *et al.* (2003) and Dalorima *et al.* (2014) emphasized the potential for trait improvement through selection in cowpea.

Components of variances

Table (6) provides comprehensive estimates of various components of variance for the studied traits, including environmental (σ^2_e), genetic (σ^2_g), and phenotypic (σ^2_p) variance, as well as genotypic (GCV) and phenotypic (PCA) coefficients of variation, GCV/PCV ratios, and broad-sense heritability (BSH).

With the exception of the number of seeds per pod, all studied traits exhibited minimal differences between phenotypic and genetic variance (Table 6). This indicates that a substantial portion of the phenotypic variance (σ^2_p) can be attributed to genetic variance (σ^2_g), emphasizing the genetic nature of the significant differences observed among the cowpea selected lines.

Analysis of the data in Table (6) reveals low discrepancies between phenotypic and genotypic variance for most studied traits, as evidenced by high GCV/PCV ratios ranging from 0.66 to 0.89. This suggests that a major proportion of the phenotypic variance (σ^2_p) is underpinned by genetic factors (σ^2_g). Furthermore, the estimated broad-sense heritability exhibited moderate to high values (ranging from 43.64% to 79.28%) across all traits, underscoring that the observed significant phenotypic differences among the studied breeding lines predominantly result from genetic factors, with minimal environmental effects on phenotypic variation, except for the trait number of seeds per pod. Consequently, the investigated traits are poised for improvement through selection based on phenotypic observations in early segregating generations. These findings align with the research of Ahmed *et al.* (2005), who documented elevated GCV and PCV for traits such as number of seeds per pod, seed yield, 100-seed weight, and pod length. Additionally, they noted high heritability for seed yield and 100-seed weight, indicating a prevalence of additive gene effects for these traits, however, the heritability for the number of days to 50% flowering was estimated at 31.83%. Similarly, Damarany (1994) and Gomes *et al.* (2021) reported high heritability values, suggesting that early-generation selection can effectively be applied for traits such as seed weight and 100-seed weight.

Assessment of polymorphism in 25th cowpea genotypes using IRAP markers

The evaluation of polymorphism within and among 25th selected cowpea genotypes, comprising 5 commercial cultivars and 20 newly developed lines, was conducted employing ten IRAP primers. The chosen primers demonstrated high efficiency, successfully amplifying bands and providing substantial information.

The amplification reactions resulted in multiple band profiles, generating 7 to 15 amplified DNA fragments per primer, with an average of 11 bands. Notably, the number of polymorphic fragments ranged from 2 to 9, averaging 1.1 polymorphic bands per primer. Primer IRAP-4375 exhibited the highest polymorphic fragments (9), while IRAP-2198 and IRAP-4351 displayed the minimum (2), as detailed in Table (7).

In total, the ten primers produced 108 reproducible fragments, of which 44 were polymorphic, indicating a considerable polymorphism level of 40.7% among the studied cowpea genotypes. The size of the amplified fragments varied between 100 and 1800 bp, as visualized in Figs. (2 & 3).

Moreover, the analysis identified unique markers capable of distinguishing between cowpea genotypes. Among the ten primers, IRAP-4352, IRAP-2198, and IRAP-2200 did not generate unique markers. In contrast, the remaining seven primers produced distinctive markers, including both unique positive and/or negative markers for cowpea genotype identification.

Notably, four IRAP primers (IRAP-2204, IRAP-4340, IRAP-4370, and IRAP-4375) generated both unique positive and negative markers, while three IRAP primers (IRAP-2197, IRAP-4351, and IRAP-3471) produced only unique positive markers. In total, twelve unique markers were identified from the ten IRAP primers, comprising eight unique positive and four unique negative markers, with molecular weights ranging from 100 to 1600 bp, as summarized in Table (7). These findings highlight the robustness of the IRAP marker system in discerning genetic variations among the cowpea genotypes studied.

Assessment of genetic relationships in 25th cowpea genotypes using IRAP markers

Understanding genetic relationships is paramount in the management of primary gene pool collections for efficient germplasm utilization in breeding and conservation programs, especially in the face of environmental changes. Molecular markers, being unaffected by environmental factors, offer a reliable estimate of genetic diversity, a crucial prerequisite for effective breeding initiatives. The calculation of genetic distances and subsequent dendrogram construction using the UPGMA method is a common practice in fingerprinting to organize germplasm efficiently and enhance genotype sampling.

In our study, we utilized IRAP marker data for 25 cowpea genotypes, creating a genetic distance tree based on Dice's genetic similarity matrix (Fig. 4). The tree revealed distinct clustering patterns, with Balady cv. forming a solitary branch, and the remaining genotypes segregating into two main clusters. Further analysis through Principal Component Analysis (PCA) scatter plots illustrated the differentiation of genotypes, highlighting unique positions for Balady and Kafr El-shaikh 1 cvs., while Qaha 1 cv. exhibited discernible distances from most other genotypes (Fig. 5).

Multivariate heatmap analysis, using the R software, reinforced the clustering observed in the genetic distance tree. Two major clusters emerged, each comprising specific genotypes. Notably, Balady cv. formed a cluster with CP 35-1, CP 23-1, CP 23, and CP 35, while Qaha 1 cv. clustered with CP 56-1, CP 65, CP 25-2, CP 56, and CP 25-3 (Fig. 6).

The findings from the IRAP analysis underscore the existing genetic differences among key cowpea varieties traded in Egyptian markets. This diversity enabled the development of promising new varieties, as evidenced by the high genetic similarity among certain new lines and Qaha 1 cv., a parent used in hybridization. Notably, CP 56-1, CP 65, CP 25-2, CP 56, and CP 25-3 demonstrated superiority in various morphological and productive traits compared to local Qaha 1 and Balady cvs., confirming their grouping in the same genetic category.

Our study aligns with the work of Sarr *et al*. (2020) and Xiong *et al*. (2016), demonstrating significant genetic variation within and among cowpea genotypes. Additionally, Dagnon *et al*. (2022) emphasized the importance of genetic diversity assessment for effective conservation programs. These results further validate the robustness of IRAP markers in discerning genetic relationships, as seen in other studies involving diverse plant species like *Medicago sativa* (Mandoulakani *et al*., 2012); Asian bamboo (Shitian *et al*., 2020); *Hordeum vulgare* (Kalendar and Schulman, 2014); *Citrus* (Abedinpour *et al*., 2014); *Lallemanti aiberica* (Cheraghi *et al*., 2018) and *O. europaea* (Khaleghi *et al*., 2017).

CONCLUSION

Based on the comprehensive analysis of the collected data, it is evident that the promising lines, namely CP 25-2, CP 25-3, CP 56, CP 56-1, and CP 65, exhibit noteworthy characteristics that make them strong contenders for certification pending further evaluations. These identified lines demonstrate not only high productivity but also exhibit favorable yield components and early maturity, coupled with the desirable seed color. The robustness of this recommendation is substantiated by genetic testing, which elucidated the specific genetic position occupied by these promising varieties within the broader spectrum of cowpea cultivars traded in Egypt. The analysis further highlighted the internal variations existing among these genotypes, underscoring their significance as

valuable sources for the development of distinct and innovative cultivars. This intrinsic diversity becomes especially crucial in addressing environmental challenges and bridging nutritional gaps, reinforcing the potential of these cultivars to contribute significantly to sustainable agricultural practices and food security.

SUMMARY

This investigation was carried out at Qaha Vegetable Research Farm, ARC, Qalyoubia Governorate, Egypt spanning from 2022 to 2023,with the aim of exploring the genetic variability and heritability of key economic characters while developing promising cowpea *(Vigna unguiculata* L.) lines. The study incorporated twenty novel lines alongside five commercially established cowpea cultivars. Notably, the results underscored that a substantial proportion of the phenotypic variance (σ^2_p) was attributable to genetic variance (σ^2_g), excluding the trait related to the number of seeds per pod. Moreover, the broad-sense heritability estimates demonstrated moderate to high values (ranging from 43.64% to 79.28%) across all scrutinized traits. This suggests that the discernible phenotypic variations among the genotypes were predominantly of genetic origin, with minimal environmental impact on the observed phenotypic diversity. Consequently, the potential for enhancing these traits through selection based on early segregating generations is highlighted. Genetic diversity of cowpea genotypes estimated using IRAP markers (Inter Retrotransposon Amplified Polymorphism). The total number of reproducible fragments amplified by the ten primers reached 108 bands, of which 44 were polymorphic fragments. This represented a level of polymorphism of 40.7%, which indicates a very high level of polymorphism among the studied cowpea genotypes. Noteworthy lines, such as CP 25-2, CP 25-3, CP 56, CP 56-1, and CP 65, exhibited promising attributes, including high productivity, favorable yield components, earliness, and desirable seed color. These lines are earmarked for potential certification pending further evaluations, showcasing their potential contribution to enhanced cowpea cultivation.

REFERENCES

Abedinpour H., Ranjbar G. A., Jelodar N. B. and Golein B., (2014). Evaluation of genetic diversity in *Citrus* genotypes by IRAP molecular marker. Int. J. Farming Allied Sci., 3 (2):230-234.

Adams B., Osekre E. A. and Amoah S., (2017). Evaluation of cowpea (*Vigna unguiculata* L.) genotypes' growth and yield performance and resistance to the cowpea seed beetle, *Callosobruchusm aculates* F. J. Exper. Agric. Inter., 19 (5): 1-9.

Agbogidi M. O., (2010). Screening six cultivars of cowpea (*Vigna unguiculata* L.) Walp for adaptation to soil contaminated with spent engine oil. J. Envir. Chem. Ecotoxicology, 2: 103-109.

Ahmed S., Zargar M. A. and Ali T., (2005). Genetic variability, heritability, genetic advance for seed yield and component

traits in cowpea. Natnl. J. Pl. Improv., 7 (2): 85-87.

Annicchiarico P., (2006). Diversity, genetic structure, distinctness and agronomic value of Italian lucerne (*Medicago sativa* L.) landraces. Euphytica, 148: 269-282.

Atta B. M., Haq M. A. and Shah T. M., (2008). Variation and inter relationships of quantitative traits in chickpea (*Cicer arietinum* L.). Pak. J. Bot., 40 (2): 637-647.

Bado B. V., Bationto A. and Cescas M., (2006). Assessment of cowpea and groundnut contributions to soil fertility and succeeding sorghum yields in the Guinean savannah zone of Burkina Faso (West Africa). Biol. Fertil. Soils, 43: 171-176.

Badr A., (2008). Molecular approaches to plant systematic and evolution. Taeckholmia, 28: 127-167.

Badr A., El-Sherif N., Aly S., Ibrahim S. D. and Ibrahim M., (2020). Genetic diversity among selected *Medicago sativa* cultivars using Inter-Retrotransposon-Amplified Polymorphism, Chloroplast DNA barcodes and morpho-agronomic trait analyses. Plants, 9 (8): 995. https://doi. org/10.3390/plants9080995.

Bhandari H. R., NishantBhanu A., Srivastava K., M. N. Singh, Shreya and Hemantaranjan A., (2017). Assessment of genetic diversity in crop plants -an overview. Adv. Plants Agric. Res., 7 (3): 279-286. https://doi.10.15406/apar.2017.07.00255.

Boukar O., Belko N., Chamarthi S., Togola A., Batieno J., Owusu E., Haruna M., Diallo S., Umar M. L. and Olufajo O., (2019). Cowpea (*Vigna unguiculata*): Genetics, genomics and breeding. Plant Breed., 138: 415-424.

Bourque G., Burns K. H., Gehring M., Gorbunova V., Seluanov A., Hammell M., Imbeault M., Izsvak Z., Levin H. L., Macfarlan T. S., Mager D. L. and Feschotte C., (2018). Ten things you should know about transposable elements. Genome Biology, 19: 199.

Burton G. W., (1952). Quantitative inheritance in grasses. Proceedings of the 6th International Grassland Cong., Pennsylvania, USA 277-283.

Cheraghi A., Rahmani F. and Hassanzadeh-Ghorttapeh A., (2018). IRAP and REMAP based genetic diversity among varieties of *Lallemanti aiberica*. Molecular Biology Res. Communications. 7 (3): 125-132. Doi. 10.22099/mbrc.2018.29924.1327.

DagnonY., Palanga K., Bammite D., Bodian A., Comlan G., Daniel F. and Koffi T., (2022). Genetic diversity and population structure of

cowpea [*Vigna unguiculata* (L.) Walp.] accessions from Togo using SSR markers. PLoS ONE, 17 (10): e0252362. https://doi.org/10.1371/journal.pone.0252362.

Dalorima T. L., Waziri A., Mohammed T. and Kyari Z., (2014). Evaluation of different varieties of cowpea (*Vigna unguiculata*) in Sudan Savanna of BornoState. The international J. sci. Technol. 2 (9): 37-42.

Damarany A. M. , (1994). Estimates of genotypic and phenotypic correlation, heritability and potency of gene set in cowpea (*Vigna unguiculata* [L.] Walp). Assuit J. Agric. Sci., 25: 1-8.

Dugje I., Omoigui L., Ekeleme F., Kamara A. and Ajeigbe H., (2009). Farmers' Guide to Cowpea Production in West Africa. IITA, Ibadan, Nigeria.

Duncan D. B., (1955). Multiple range and multiple F test. Biometrics, 11: 1-42.

El-Shazly H. H., HAhmed I. S., Hamouda M. M. and Badr A., (2020). Genetic diversity and population structure of the medicinal plant *Achillea fragrantissima* (Forssk.) Sch. Bip. in the mountains of South Sinai, Egypt. Plant Gene, 21: 100212. doi.org/10.1016/j.plgene. 2019. 100212.

Farouji A. E., Khodayari H., Saeidi H. and Rahiminejad M. R., (2015). Genetic diversity of diploid *Triticum* species in Iran assessed using inter-retroelement amplified polymorphisms (IRAP) markers. Biology, 70: 52-60.

Gbadegesin M. A. and Beeching J. R., (2010). Enhancer/Suppressor mutator (En/Spm)-like transposable elements of cassava (*Manihot esculenta*) are transcriptionally inactive. Genet. Mol. Res.9: 639-650. http://dx. doi.org/10.4238/vol9-2gmr713.

Gnanamurthy S., Mariyammal S., Dhanavel D. Bharathi and T., (2012). Effect of gamma rays on yield and yield components characters R3 generation in cowpea (*Vigna unguiculata* (L.). Walp.). Inter. J. Res. Plant Sci., 2: 39-42.

Gomes A. M. F., Draper D., Nhantumbo N., Massinga R., Ramalho J. C., Marques I. and Ribeiro-Barros A. I., (2021). Diversity of cowpea [*Vigna unguiculata* (L.) Walp] landraces in Mozambique: New opportunities for crop improvement and future breeding programs. Agronomy, 11 (1): 1-12.

Gomez A. K. and Gomez A. A., (1984). Statistical Procedures for Agricultural Research. 2nd edition & Sons Pub., PP.139-153.

Hussein A. H. and Abd El-Hady M. A. H., (2015). A comparison of some promising lines and commercial cultivars of cowpea. Egypt. J. Plant Breed. 19 (1): 101-109.

Johnson H. W., Robinson H. F. and Comstock R. E., (1955). Estimates of genetic and environmental variability in soybeans. Agronomy J., 47: 314-318.

Ibro G., Sorgho M. C., Idris A. A., Moussa B., Baributsa D. and Lowenberg-DeBoer J., (2014). Adoption of cowpea hermetic storage by women in Nigeria, Niger and Burkina Faso. J. Stored Products Res., 58: 87-96.

Jayathilake C., Visvanathan R., Deen A., Bangamuwage R., Jayawardana B. C., Nammi S. and Liyanage R., (2018) Cowpea: An overview on its nutritional facts and health benefits. J. Sci. Food Agric., 98: 4793-4806.

Kalendar R. and Schulman A. H., (2007). IRAP and REMAP for retrotransposon-based genotyping and fingerprinting. Nat. Protoc., 1 (5): 2478-2484.

Kalendar R. and Schulman A. H., (2013). Transposon-Based Tagging: IRAP, REMAP, and iPBS. Adv. Struct. Saf. Stud. 1115: 233-255.

Kalendar R. and Schulman A. H., (2014).Transposon-based tagging: IRAP, REMAP, and iPBS. In: "Molecular Plant Taxonomy. Methods in Molecular Biology(Methods and Protocols)", Besse, P. (Ed.), Vol 1115. Humana Press, Totowa, NJ.

Kalendar R., Antonius K., Smykal P. and Schulman A. H., (2010). IPBS: A universal method for DNA fingerprinting and retrotransposon isolation. Theor. Appl. Genet.,121 (8):1419-1430. doi:10.1007/s00122-010-1398-2.

Khaleghi E., Sorkheh K., Chaleshtori M. H. and Ercisli S., (2017). Elucidate genetic diversity and population structure of *Olea europaea* L. germplasm in Iran using AFLP and IRAP molecular markers. 3 Biotech., 7: 71. doi: 10.1007/s13205-017-0669-x.

Kumar A. and Bennetzen J. L., (1999). Plant Retrotransposons. Annu. Rev. Genet., 33: 479-532.

Lopes F. C. C., Gomes R. L. F. and Filho F. R. F., (2003). Genetic control of cowpea seed sizes. Scientia Agricola, 60 (2): 315-318.

Mandoulakani B. A., Piri Y., Darvishzadeh R., Bernoosi I. and Jafari M., (2012). Retro element in sertional polymorphism and genetic diversity in *Medicago sativa* populations revealed by IRAP and REMAP markers. Plant Mol. Bio. Rep., 30 (2): 286-296.

Maniatis T., Fritsch E. F. and Sambrook J., (1988). Molecular Cloning: A Laboratory Manual, 2nd ed.; Cold Spring Harb: New York, USA.

More A. D. and Borkar A. T., (2016). Analysis of genetic variability, heritability and genetic advance in *Phaseolus vulgaris* L. Inter. J. Current Microbio. App. Sci., 5: 494-503.

Nasri S., Mandoulakani B. A., Darvishzadeh R. and Bernousi I., (2013). Retrotransposon insertional polymorphism in Iranian bread wheat cultivars and breeding lines revealed by IRAP and REMAP markers. Biochem. Genet., 51: 927-943.

Padulosi S. and Ng N. Q., (1997). Origin, taxonomy and morphology of *Vigna unguiculata* (L.) Walp. Advances in cowpea research. Inter. Inst. Tropical Agric. (IITA) and Japan Inter. Res. Center Agric. Sci. (JIRCAS), Ibadan, Nigeria, pp. 1-12.

Rangel A., Domont G. B., Pedrosa C. and Ferreira S. T., (2003). Functional properties of purified vicilins from cowpea (*Vigna unguiculata*) and pea (*Pisum sativum*)and cowpea protein isolate. J. Agric. Food Chem., 51:5792-5797.

Sarr A., Bodian A., Gbedevi K. M., Ndir K. N. and Ajewole O. O., (2020). Genetic diversity and population structure analyses of wild relatives and cultivated cowpea (*Vigna unguiculata* (L.) Walp.) from Senegal using simple sequence repeat markers. Plant Mol. Biol.https://doi.org/ 10.1007/s11105-020-01232-z.

Scarano D., Rubio F., Ruiz J. J., Rao R. and Corrado G., (2014). Morphological and genetic diversity among and within common bean (*Phaseolus vulgaris* L.) landraces from the Campania region (Southern Italy). ScientiaHorticulturae, 180:72-78.

Shehata M., Sayed L., Badawy F. and Fahmy E., (2015). Assessment of genetic diversity in yeast and barley by retrotransposon-based molecular markers. Egyptian J. Gene. Cytology, 44: 371-385.

Shitian L., Muthusamy R., Kunnummal K. V., Ruslan K., Kim Y. and Mingbing Z., (2020). Development and deployment of high-throughput retrotransposon-based markers reveal genetic diversity and population structure of Asian Bamboo. Forests, 11 (1): 31. https://doi.org/ 10.3390/f11010031.

Singh B. B., Chambliss O. L. and Sharma B., (1997). Recent advances in cowpea. In: B. B. Singh, D. R. Mohan Raj, K. E. Dashiel and L. E. N. Jackai (eds.). Adv. Cowpea Res. Co-publication of Inter. Inst. Tropical Agric. (IITA) and Japan Inter.

Res. Center Agric. Sci. (JIRCAS), Ibadan, Nigeria, 30-49.

Singh R. K. and Chaudhary B. D., (1995). Biometrical Methods in Quantitative Genetic Analysis. Kalyani Publishers, New Delhi, India.

Somasundaram M., Subbaraya U. S., Ramaraj S., Palani D., Mustaffa M. M., Kalaimughilan K. and Chandrasekar A., (2023). Inter retrotransposon based genetic diversity and phylogenetic analysis among the Musa germplasm accessions. J. Plant Biochem. Biotech. 29: 114-124. https://doi.org/10.1007/s13562-019-00519-x.

Steel R. C. D. and Torrie J. H., (1981). Principles and Procedures of Statistics. McGraw-Hill, New York, USA.

Taheri M. T., Alavi-Kia S. S., Mohammadi S. A. and Vahed M. M., (2018). Assessment of genetic diversity and relationships among *Triticum urartu* and *Triticum boeoticum* populations from Iran using IRAP and REMAP markers. Genet. Resour. Crop. Evol., 65: 1867-1878.

Xiong H., Shi A., Mou B., Qin J., Motes D. and Lu W., (2016). Genetic diversity and population structure of cowpea (*Vigna unguiculata L. Walp*). PLoS One., 4:1-15. https://doi.org/10.1371/journal.pone.0160941.

Table (1): Assessed cowpea genotypes throughout the2022 and 2023 seasons, along with their distinctive characteristics.

#	Genotype	Source	Growth habit	Flower colour	Seed color	Distinctive characters
1	**CP 23**	Qaha 1 X Balady	Erect	Purple	Creamy with brown eye	Small-sized seeds
2	**CP23-1**	Qaha 1 X Balady	Erect	Purple	Brown	Early maturity
3	**CP 25**	Qaha 1 X Balady	Erect	White	White with brown eye	Early maturity, small-sized seeds
4	**CP 25-2**	Qaha 1 X Balady	Erect	White	Creamy with brown eye	High yielding, early maturity, small-sized seeds
5	**CP 25-3**	Qaha 1 X Balady	Erect	Purple	brown	High yielding, early maturity, large-sized seeds
6	**CP 30-1**	Qaha 1 X Balady	Erect	White	Creamy with brown eye	Large-sized seeds
7	**CP 35**	Qaha 1 X Balady	Semi-erect	Purple	Brown	High number of seeds/pod
8	**CP 35-1**	Qaha 1 X Balady	Semi-erect	Pink	Creamy with brown eye	Early maturity
9	**CP 52**	Qaha 1 X Tiba	Erect	White	White with brown eye	Good seed color
10	**CP 52-1**	Qaha 1 X Dokki 331	Erect	White	brown	Good seed color
11	**CP 56**	Qaha 1 X Dokki 331	Erect	White	Creamy with brown eye	High yielding
12	**CP 56-1**	Qaha 1 X Dokki 331	Trailing	Purple	Creamy with brown eye	High yielding, early maturity, large-sized seeds
13	**CP 57**	Qaha 1 X Dokki 331	Erect	Dark purple	Black	Early maturity, small-sized seeds
14	**CP 64**	Qaha 1 X Dokki 331	Erect	White	White with Black eye	Early maturity
15	**CP 65**	Qaha 1 X Dokki 331	Semi-erect	White	Creamy with brown eye	High yielding, early maturity, large-sized seeds
16	**CP 65-1**	Qaha 1 X Dokki 331	Semi-erect	Purple	Brown	Early maturity, large-sized seeds
17	**CP 66**	Qaha 1 X Dokki 331	Erect	White	White with Black eye	High yielding, large-sized seeds
18	**CP 67**	Segregation from cv. Qaha 1	Erect	White	White with Black eye	Early maturity, large-sized seeds
19	**CP 67-1**	Segregation from cv. Qaha 1	Erect	White	Brown	Small-sized seeds
20	**CP 70**	Segregation from cv. Tiba	Erect	White	Brown	Large-sized seeds
21	**Balady**	Landrace	Trailing	Dark purple	brown	High yielding, small-sized seeds,
22	**Cream 7**	HRI[z]	Erect	White	Creamy	Late maturity
23	**Kafr El-shaikh 1**	HRI	Erect	White	Creamy	Late maturity, large-sized seeds
24	**Qaha 1**	HRI	Erect	White	Creamy with brown eye	Early maturity, small-sized seeds
25	**Tiba**	HRI	Erect	White	Creamy with brown eye	High yielding, early maturity

HRI[z]: Horticultural Research Institute

Table (2): The sequence information for the 10 primers used in the IRAP-PCR marker assay.

#	Primers	Sequence 5’ to 3’
1	IRAP4352	ACCCGGAAGGGCGGTTCATGCAA
2	IRAP-2198	ATCCTTCGCGTAGATCAAGCGCCA
3	IRAP 2197	GAAGTACCGATTTACTTCCGTGTA
4	IRAP 2200	ATGTGACAGTCGACTAACCAC
5	IRAP 2204	TACCCTTTTAAGGGATCAACC
6	IRAP 4351	AACTTGATCCAGATCATCTCC
7	IRAP 4340	ATGGTTGTCGAAACTCCAGC
8	IRAP 4370	ATGCCGTATTCTCAGCATCC
9	IRAP 4375	ATCGCTCCGGGTGCCTAACAC
10	IRAP 3471	ATCGCTCCGGGTGCCTAACAC

Table (3): IRAP -PCR reaction parameters.

	Temperature	Time period	Cycle
Initial denaturation	94ºC	5min	1
Denaturation	94ºC	50 Sec	
Annealing	54ºC	55 sec	35
Extension	72ºC	1.3 min	
Final extension	72ºC	10 min	1

Table (4): Mean performance of genotypes for number of days to flowering, pod length andnumber of seeds per pod characters.

Genotypes	Characters								
	Number of days to flowering			Pod length (cm)			Number of seeds/pod		
	2022	2023	Combined	2022	2023	Combined	2022	2023	Combined
CP 23	55.67 a-d	53.67 d-f	54.67 b-d	13.29 jk	12.54 g	12.94 I	10.70 b	10.53 bc	10.62 de
CP23-1	49.00 g	47.67 i	48.33 j	14.82 f-j	15.33 c-f	15.08 d-h	12.07 ab	12.53 a	12.30 ab
CP 25	52.00 d-g	50.67 gh	51.33 g-i	12.50 kl	17.09 ab	14.80 e-i	12.00 ab	12.50 ab	12.25 a-c
CP 25-2	52.67 d-g	50.67 gh	51.67 f-i	11.73 l	16.02 a-e	13.87 hi	11.33 ab	11.97 a-c	11.65 b-e
CP 25-3	51.00 e-g	49.67 hi	50.33 i	19.16 a	16.91 a-c	18.04 a	12.67 ab	12.27 a-c	12.47 ab
CP 30-1	54.00 b-f	52.67 e-g	53.33 c-f	16.49 b-e	16.09 a-e	16.29 a-e	10.83 b	10.50 c	10.67 c-e
CP 35	54.00 b-f	52.67 e-g	53.33 c-f	15.61 d-h	16.19 a-e	15.90 b-h	11.43 ab	12.00 a-c	11.72 b-e
CP 35-1	51.67 d-g	50.67 gh	51.17 g-i	15.10 e-i	16.06 a-e	15.58 c-h	11.53 ab	11.73 a-c	11.63 b-e
CP 52	55.00 a-e	53.67 d-f	54.33 b-e	16.87 b-d	16.03 a-e	16.45 a-e	12.13 ab	11.10 a-c	11.62 b-e
CP 52-1	57.67 ab	57.67 ab	57.67 a	16.88 b-d	15.01 d-f	15.95 b-g	11.33 ab	10.80 a-c	11.07 b-e
CP 56	54.00 b-f	52.67 e-g	53.33 c-f	14.30 h-j	16.51 a-d	15.41 d-h	11.40 ab	11.43 a-c	11.42 b-e
CP 56-1	51.67 d-g	50.67 gh	51.17 g-i	14.60 g-j	14.99 d-f	14.80 e-i	11.23 b	10.77 a-c	11.00 b-e
CP 57	53.00 c-g	50.67 gh	51.83 f-i	13.98 i-k	14.33 f	14.16 f-i	10.43 b	10.53 bc	10.48 e
CP 64	52.33 d-g	52.67 e-g	52.50 e-h	15.37 d-i	15.33 c-f	15.35 d-h	10.47 b	10.50 c	10.48 e
CP 65	52.00 d-g	50.67 gh	51.33 g-i	15.90 d-g	15.19 d-f	15.55 c-h	11.80 ab	12.07 a-c	11.93 a-e
CP 65-1	51.33 e-g	50.67 gh	51.00 g-i	15.93 d-g	15.17 d-f	15.55 c-h	11.50 ab	12.07 a-c	11.78 b-e
CP 66	59.00 a	58.67 a	58.83 a	16.18 c-f	15.66 b-f	15.92 b-g	11.77 ab	11.47 a-c	11.62 b-e
CP 67	50.67 fg	51.00 f-h	50.83 hi	16.56 b-e	17.49 a	17.03 a-d	12.20 ab	12.27 a-c	12.23 a-d
CP 67-1	55.00 a-e	55.67 b-d	55.33 b	15.84 d-g	16.30 a-e	16.07 a-f	12.33 ab	12.33 a-c	12.33 ab
CP 70	57.00 a-c	58.67 a	57.83 a	16.33 c-f	17.00 a-c	16.65 a-e	11.87 ab	12.53 a	12.20 a-d
Balady	55.67 a-d	54.67 c-e	55.17 bc	13.37 jk	14.67 ef	14.02 g-i	10.70 b	11.07 a-c	10.88 b-e
Cream 7	58.33 a	56.67 a-c	57.50 a	17.98 ab	17.05 ab	17.52 a-c	11.60 ab	11.80 a-c	11.70 b-e
Kafr Elshaikh 1	59.00 a	58.67 a	58.83 a	17.57 bc	17.69 a	17.63 ab	14.20 a	12.73 a	13.47 a
Qaha 1	51.00 e-g	50.67 gh	50.83 hi	16.43 c-e	16.48 a-d	16.46 a-e	11.07 b	11.50 a-c	11.28 b-e
Tiba	53.00 c-g	52.67 e-g	52.83 d-g	16.10 c-g	16.13 a-e	16.12 a-f	12.07 ab	12.43 a-c	12.25 a-c

Means with the same letter are not significantly different from each other

Table (5): Mean performances of selected cowpea lines and check cultivars for 100 seed weight and seed yield/fed. characters.

Genotypes	Characters					
	100- seed weight			Seed yield/fed. (ton/fed.)		
	2022	2023	Combined	2022	2023	Combined
CP 23	13.97 e-g	14.03 f-j	14.00 f-j	0.464 i	0.403 h	0.434 j
CP23-1	15.45 c-e	14.16 f-j	14.81 d-i	0.756 e-g	0.780 c-g	0.768 d-f
CP 25	12.85 gh	14.60 d-i	13.72 f-j	0.755 fg	0.735 c-h	0.745 e-g
CP 25-2	13.37 f-h	12.82 h-k	13.09 g-j	1.032 bc	1.086 bc	1.059 b
CP 25-3	18.31 ab	15.11 c-g	16.71 a-e	1.002 bc	0.983 cd	0.993 bc
CP 30-1	18.79 a	15.52 c-g	17.15 a-d	0.598 g-i	0.593 e-h	0.596 f-j
CP 35	15.54 c-e	13.79 f-j	14.66 e-i	0.752 fg	0.765 c-h	0.758 d-f
CP 35-1	14.70 d-f	13.51 g-k	14.11 f-j	0.561 hi	0.584 e-h	0.573 g-j
CP 52	15.61 c-e	14.36 e-j	14.98 c-g	0.467 i	0.451 gh	0.459 j
CP 52-1	16.20 cd	14.99 c-h	15.59 b-f	0.542 hi	0.525 f-h	0.533 ij
CP 56	15.42 c-e	18.47 a	16.94 a-e	0.933 b-e	0.920 c-e	0.927 b-d
CP 56-1	18.93 a	15.71 c-g	17.32 a-c	0.941 b-d	0.989 cd	0.965 bc
CP 57	12.18 h	11.47 k	11.83 j	0.544 hi	0.583 e-h	0.564 h-j
CP 64	15.22 c-e	14.50 d-j	14.86 d-h	0.491 i	0.473 gh	0.482 j
CP 65	18.76 a	15.25 c-g	17.01 a-e	1.449 a	1.464 a	1.456 a
CP 65-1	18.37 a	15.00 c-h	16.68 a-e	0.469 i	0.477 gh	0.473 j
CP 66	18.09 ab	17.20 a-c	17.65 ab	0.966 bc	0.987 cd	0.976 bc
CP 67	19.26 a	16.76 a-d	18.01 a	0.871 c-f	0.864 c-f	0.868 c-e
CP 67-1	11.84 h	12.55 i-k	12.20 j	0.766 d-g	0.768 c-h	0.767 d-f
CP 70	16.67 bc	16.49 a-e	16.58 a-e	0.537 hi	0.550 e-h	0.543 ij
Balady	12.53 gh	12.30 jk	12.42 ij	1.088 b	1.073 bc	1.080 b
Cream 7	15.43 c-e	12.37 i-k	13.90 f-j	0.671 gh	0.674 d-h	0.672 f-i
Kafr Elshaikh 1	16.33 cd	18.07 ab	17.20 a-d	0.539 hi	0.571 e-h	0.555 ij
Qaha 1	12.30 gh	12.83 h-k	12.56 h-j	0.747 fg	0.722 c-h	0.735 e-h
Tiba	15.83 cd	15.87 b-f	15.85 a-f	1.428 a	1.433 ab	1.431 a

Means with the same letter are not significantly different from each other

Table (6): Variance components (σ^2_p,σ^2_gandσ^2_e), genotypic (GCV) and phenotypic (PCA) coefficient of variation and broad sense heritability (BSH%) for cowpea traits.

Variance Compo-nents	Characters				
	No. days to flowering	**Pod length**	**No. seeds/ pod**	**100 seeds weight**	**Seed yield /fed.**
ó^2_e	2.303	0.509	0.500	0.923	0.025
ó^2_g	8.816	1.094	0.387	2.999	0.073
ó^2_p	11.119	1.603	0.887	3.922	0.098
H^2_{Bs} (%)	79. 28	68.25	43. 64	76. 46	74. 87
G.C.V. (%)	0.0036	0.0163	0.0187	0.0294	1.6937
P.C.V. (%)	0.0041	0.0198	0.0284	0.0336	1.9574
G.C.V/ P.C.V	0.89	0.83	0.66	0.87	0.87

Table (7): Levels of polymorphism, total number of bands, monomorphic bands, polymorphic bands, percentage of polymorphism, unique positive and unique negative bands as revealed by IRAP markers among the 25th cowpea genotypes.

No.	Primers	Total number of bands	Mono morphic bands	Poly morphic bands	% p	UPM	MW bp	UNM	MW bp
1	**IRAP4352**	7	4	3	43	0		0	
2	**IRAP-2198**	9	7	2	22	0		0	
3	**IRAP 2197**	12	8	4	33	2(L5)	600, 700	0	
4	**IRAP 2200**	13	9	4	31	0		0	
5	**IRAP 2204**	9	5	4	44	1(L14)	150	1(CV25)	180
6	**IRAP 4351**	14	12	2	14	1(L8)	150	0	
7	**IRAP 4340**	11	4	7	64	1(L4)	1600	1(CV23)	1400
8	**IRAP 4370**	8	3	5	63	1(L4)	900	1(1)	180
9	**IRAP 4375**	15	6	9	60	1(L9)	150	1(CV25)	220
10	**IRAP 3471**	10	6	4	40	1(L8)	1600	0	
Total		**108**	**64**	**44**	**407**	**8**		**4**	
Average		**10.8**	**6.4**	**4.4**	**40.7**	**0.8**		**0.4**	

Fig. (1): Illustrates the compilation of cowpea genotypes examined during the 2022 and 2023 seasons. Genomic DNA extraction, purification and quantification of 25th cowpea genotypes.

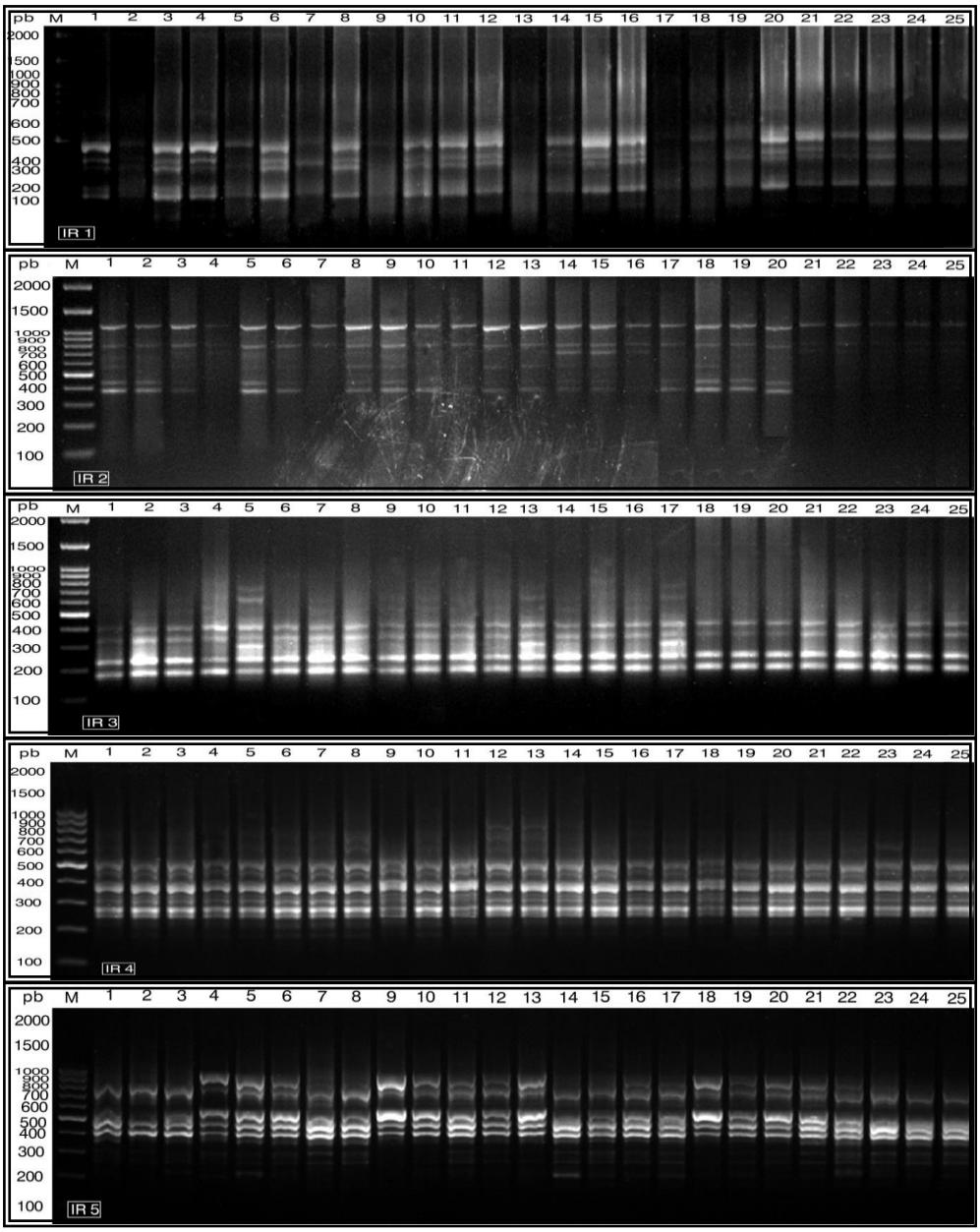

Fig. (2): IRAP profiles of 25th cowpea genotypes (1 - 25) as detected with primers (1) IRAP4352, (2) IRAP-2198, (3) IRAP 2197, (4) IRAP 2200 and (5) IRAP 2204. DNA molecular weight standards (M) 100 bp DNA ladder.

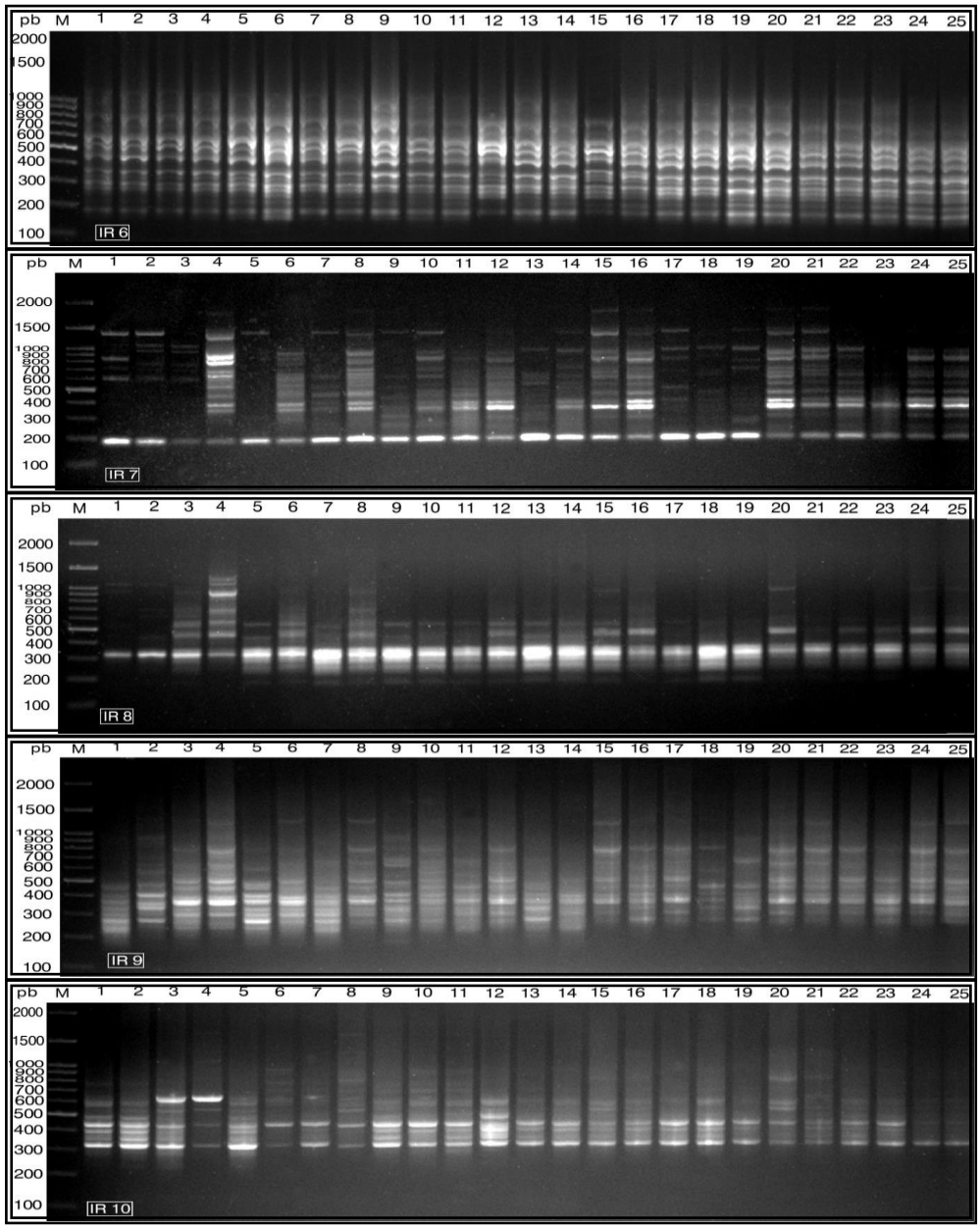

Fig. (3): IRAP profiles of 25th cowpea genotypes (1 - 25) as detected with primers (6) IRAP 4351, (7) IRAP 4340, (8) IRAP 4370, (9) IRAP 4375 and (10) IRAP 3471. DNA molecular weight standards (M) 100 bp DNA ladder.

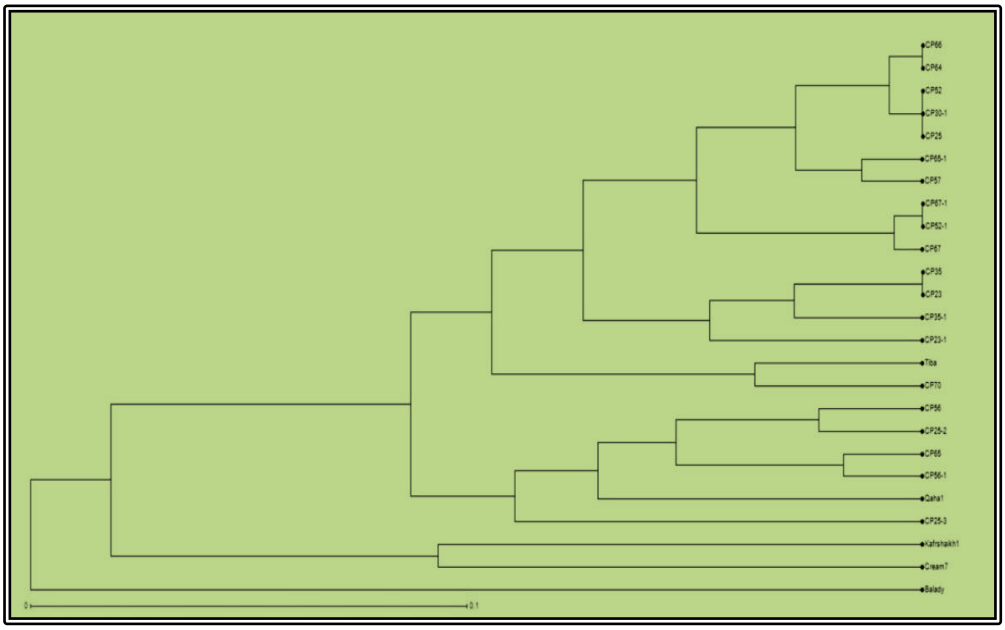

Fig. (4): Cluster tree illustrating the relationship of 25th cowpea genotypes based on the analysis ofIRAP marker polymorphism, constructed using the Euclidean similarity matrices computed as Dicecoe_cients and using the unweighted pair group method with arithmetic mean (UPGMA) algorithmin the PAST software.

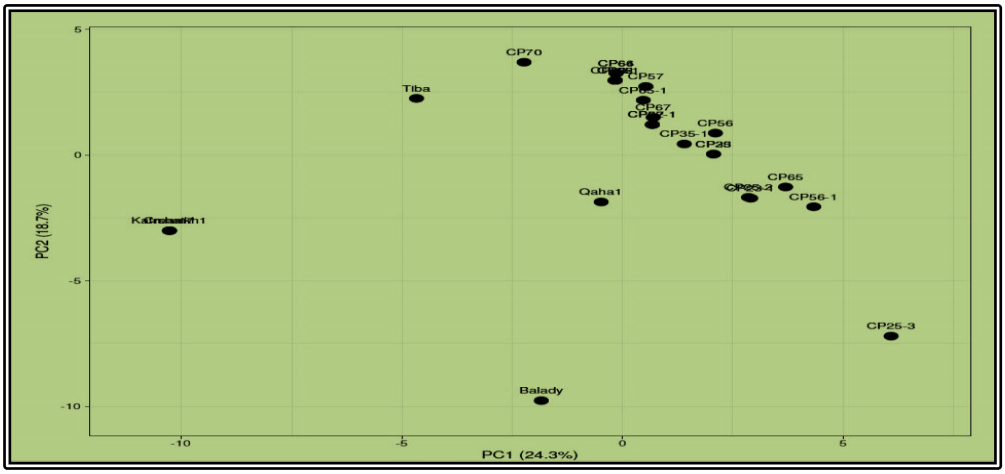

Fig. (5): The PCA analysis (principle component analysis) scatter diagram illustrating the genetic diversityexpressed by the grouping of of 25th cowpea genotypes based on the analysis of IRAP marker polymorphism and by blotting the first two principale components using PAST software.

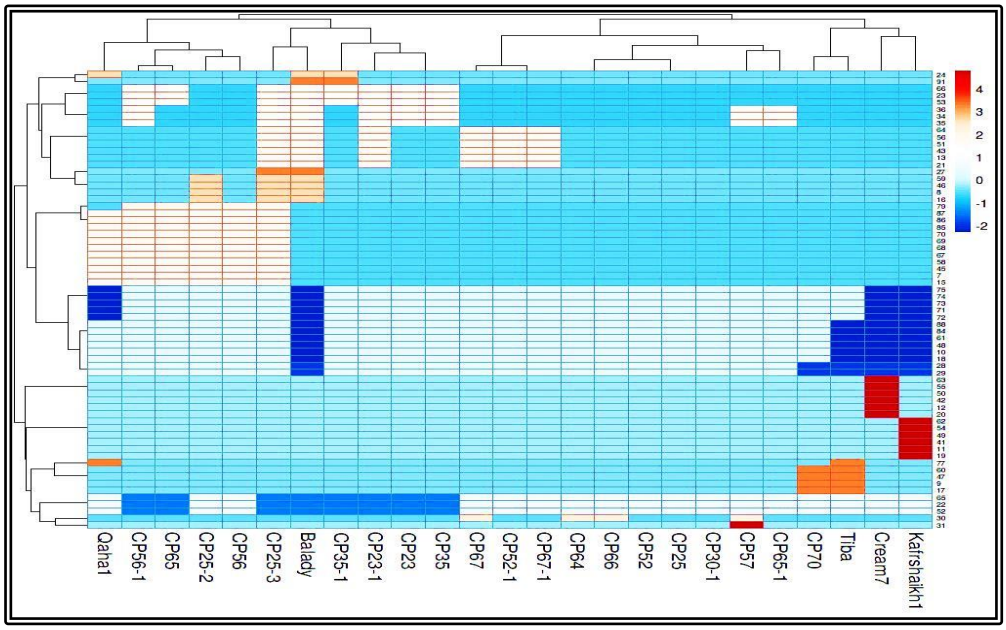

Fig. (6): Multivariate heatmap illustrating the genetic diversity of of 25th cowpea genotypes based on the IRAP markers constructed using the module of heatmap of R software.

MUTATIONAL ANALYSIS OF *BRAF* GENE IN EGYPTIAN HEP ATOCELLULAR CARCINOMA PATIENTS USING *NGS*

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Hepatocellular carcinoma HCC is the fourth highest cause of cancer-related deaths globally. Cirrhosis caused by persistent infection with the hepatitis B or C virus accounts for 80-90% of HCC cases. Many individuals with HCC are not candidates for potentially curative therapy such as surgical resection and transplantation due to their advanced stage of the disease Russo *et al*. (2022).

BRAF is a protein kinase that targets serine and threonine residues. Mutations in this gene have also been linked to a variety of malignancies, including colorectal cancer, non-Hodgkin lymphoma, thyroid carcinoma, malignant melanoma, non-small cell lung carcinoma, and lung adenocarcinomas. *BRAF* is one of the essential cancer-associated genes in this pathway Gnoni *et al*. (2019). Cancer patients with abnormally activated RAS/RAF signaling pathways tend to have a bad prognosis. An innovative approach to treating HCC involves focusing on the RAS/RAF pathway. The RAF kinase inhibitor sorafenib helps treat HCC, so *BRAF* mutations are now the go-to target for HCC treatment. The therapy of advanced HCC has found a promising target in *BRAF* mutations Pope *et al*. (2021). The objective of the present investigation was to examine the relationship between *BRAF* and the progression of HCC in Egyptian HCC patients through the utilization of NGS technology.

MATERIALS AND METHODS

a. Study design and participants

This research comprised 21 HCC patients (eighteen males along with three females, having average ages of 62 years) recruited prospectively from the inpatient and outpatient clinics of the oncology unit at Egypt's Liver National Institute-Menoufia University.

All HCC patients had a family history taking, a clinical examination, tumor staging, and an exhaustive list of laboratory testing (liver enzymes, coagulation profile, renal function profile, and CBC) and chest X-ray.

The study was performed from January to November 2020, under the permission of Menoufia University's Ethics Committee (National Liver Institute). The study did not include any other cancer patients.

b. Sample collection and cell-free DNA extraction:

After collecting peripheral blood samples (1-3 mL) in EDTA-containing tubes, genomic DNA was isolated from a whole blood sample, and the plasma was frozen at -80ºC for cell-free DNA extraction. The QIAamp® DSP Virus spin kit Version 1 (QIAGEN, Hilden, Germany) was used to extract circulating cell-free DNA from plasma, as recommended by the manufacturer.

c Next-generation sequencing

Following the manufacturer's instructions, cell-free DNA was extracted from plasma samples using the QIAamp® DSP Virus spin kit Version 1 (QIAGEN, Hilden, Germany). Using the Gene JET Genomic DNA Purification Kit (Thermoscientific, Cat#K0), genomic DNA was extracted. Ion AmpliSeq HiFi Master Mix (Ion AmpliSeqTM Li-brary kit 2.0, Thermo Fisher Scientific, Inc.) and the Ion AmpliSeqTM Cancer Hotspot Panel (version 2) were used to amplify 10 ng of DNA for library preparation. Following the directions provided by the manufacturer, the library was quantified using qPCR with the ion library TaqMan® Quantitation Kit (Thermo Fisher Scientific, Inc.). Life Technologies' Ion OneTouchTM2 system was updated and installed on the templates. Ten percent to thirty percent of the ISPs produced were template positive; this was checked using the Ionosphere quality control kit made by Thermo Fisher Scientific, Inc. The template ISPs were loaded onto Ion 316TM chips after enrichment and sequenced using the IonPGMTM Sequencing Hi-Q view kit v2 and PGMTM (Life Technologies) according to the manufacturer's instructions.

d. Bioinformatics data analysis

To examine both normal and tumor samples, the ion ampliseq custom panel approach was used using the default plugin settings in Thermo Fisher's Ion

Reporter server 5.10. We used Torrent Suite (version 3.6.2; Thermo Fisher Scientific, Inc.) to compare the data to Human Genome Version 19 (hg19). Thermo Fisher Scientific, Inc.'s Coverage Analysis plug-in (version 3.6) was used. Allele frequency more than 10%, general uniformity greater than 80%, quality greater than 20, and average base coverage greater than 500x readings were the cutoffs. A plug-in called Variant Caller (version 3.6; Thermo Fisher Scientific, Inc.) was used to detect mutations. The Integrated Genome Viewer IGV at the Broad Institute was used to verify each mutation (www.broadinstitute.org).

Statistical analysis

Frequencies and percentages were used to represent data from categorical variables, whereas mean Standard Deviation or median (IQR) was used to represent data from continuous variables. The significance between categorical variables was examined using the Chi-square test, while continuous variables were tested using Mann-Whitney U tests. $P<0.05$ was established as the criterion for statistical significance.

RESULTS AND DISCUSSION

The study population comprised of 18 (85.7%) males and 3 (14.3%) females. Of these, 13 (61.9%) were <60 years old, and 8 (38.1%) were ≥60 years old, with a mean age (of 62.19 ±8.85) and a median (of 63) years. A total of 19 patients had HCV and 1 patient had HBV, 13 (61.9%) had bilharzia antibodies. Over 47.6% of HCC patients had co-morbidities, diabetes (33.3%) and hypertension (14.3%) were among the common co-morbid conditions, (Table 1).

Mutant *BRAF* patients were significantly more likely to be older age >60 years (90%). BRAF gene mutations were also significantly more likely to be without family history (90%). All cases with muted BRAF had HCV (100%), 90% had no ascites, 10% had positive PVI, and 10% had lung metastasis. All pathological features were not significant, (Table 2).

A recent study found that 85.7% of HCC patients are men. The findings of the analysis of the influence of sex disparities on disease outcomes are inconclusive Braunwarth *et al*. (2020) and Rich *et al*. (2020). Increased exposure to risk factors, androgens (AR), and estrogens (ER), as well as male predominance in HCC Zhang *et al*. (2020). According to the current study, individuals 60 years old or older make up 61.9% of those with HCC. As a result, a variety of causes, including race, ethnicity, and genetic predisposition, could be implicated in the age disparity Mak and Kramvis (2021).

According to other studies, smoking has several harmful consequences on the liver, such as liver carcinogens Li *et al*. (2019). Hence, there was no statistically significant correlation between smoking and HCC. Increasing research indicates that HCC risk with an aggressive nature is significantly increased by a family history of liver

cancer Loomba *et al*. (2013) and Caruso *et al*. (2017).

In this study, 61.9% of the 21 HCC patients had bilharzia antibodies. Our findings are consistent with those of researchers, who found Schistosoma antibodies in 67.7% of Egyptian HCC patientsRamadan *et al*. (2021). In this study, bilharzia was identified as HCC risk factor (OR=1.625, 95% CI 0.558-4.73).

In this study, 90.5% of HCC patients had anti-HCV antibodies. HCV infection was linked to a 9.5-fold increased risk of HCC (OR= 9.50, 95% CI 1.96–46.01). Whereas only 4.7% of people tested positive for HBV (OR= 0.050, 95% CI 0.006-0.407). The study's findings are in line with those of earlier research, which found that HCV infection is the main cause of cirrhosis (93%) and a risk factor for HCC Mohamed *et al*. (2015) and Rashed *et al*. (2020).

In the current study, the mean serum AFP level was 2417.07± 9230.79, the median was 42 ng/dL as shown in (Table 3). According to Zhang *et al*. (2020), a blood AFP level of 400 ng/dL provides the highest sensitivity and specificity for detecting HCC.

The current study found a statistically significant relationship between ascites and HCC patients (P=0.023). This finding is consistent with Hsu *et al*. (2013) who found that 23% of patients had ascites at the time of diagnosis. Although the prevalence of macroscopic PVI varies across studies and is present in 30% to 62% of instances with advanced HCC, it is unquestionably underreported Shehta *et al*. (2021).

In a previous study, 14.3% of participants had a positive PVI that was significantly correlated with HCC (P = 0.01), Brain (2%), peritoneum/omentum (11%), adrenal glands (11%), bone (28%), local lymph nodes (53%), and lung (55%) are the most frequent extrahepatic HCC metastatic locations to their frequencies Becker *et al*. (2014).

Extrahepatic metastasis is a sign of advanced HCC, according to clinical standards. The lymph nodes and the lungs in this study were metastatic sites (14.3% for each). The classic Child-Pugh rating system has been the most popular way to evaluate liver function and determine the effectiveness of treatments for many years Zhao *et al*. (2020).

In 21 HCC patients, Child's A had a 76.2% preponderance, followed by Child's B with 14.3% and Child's C with 9.5%. This study's findings are consistent with those of Hassan-Kadle *et al*. (2022), who noted that 73.6% of patients were classified as Child's A, 17.2% as Child's B, and 9.0% as Child's C in the Child-Pugh classification. Patients with diabetes mellitus (DM), whose incidence is steadily rising globally, are two to three times more likely to develop HCC Li *et al*. (2017).

In the present study, 33.3% of participants had DM that was not

significantly associated. HCC mortality has been related to primary hypertension, however, the reasons underlying this association are not fully understood Lopez-Lopez *et al*. (2020). In this study, 14.3% of 21 HCC patients had hypertension, and this association was significant (P = 0.01).

Various staging systems, including the BCLC staging system, have been proposed in recent years Hsu *et al*. (2013). The Child-Pugh score, tumor burden, and patient performance status are only a few of the factors the BCLC staging system considers. According to the study population's CT scan results, 81.0% of the 21 cases of HCC detected by CT scan had large tumors measuring more than 5 cm in diameter. Among these cases, 42.9% had a single lesion and 57.1% had multiple lesions. The BCLC staging revealed that stages A and C (33.35% for each) were more prevalent.

In this study, we investigated the prevalence of *BRAF* genetic alterations in a cohort of 21 human HCC patients. For the *BRAF* gene, somatic mutations were frequently found in 10 patients from 21 patients (47.6%).

Previous work reported the *BRAF* gene mutation in 65 HCC patients and reported that among 65 cases, the oncogenic mutations were detected in 15 (23%) patients for the *BRAF* gene Colombino *et al*. (2012).

In all 21 patients who underwent *BRAF* gene sequencing, mutations were identified in 10/21 (47.6%) of samples. There were 10 variants, out of them 8/10 (80%) were single nucleotide variants (SNVs), 1/10 (10%) were copy number variants (CNVs) and 1/10 (10%) were insertions/deletion variants (INDELs). Genomic variant annotation and filtering were further interpreted using the Ensembl Variant Effect Predictor (VEP). The program uses the Ensembl/GENCODE or RefSeq gene sets to forecast the molecular effects of variants. Among SNVs, 71.4% (5/7) were novel somatic mutations (missense variant), and 28.6% (2/7) were existing germline mutations (coding transcript intronic variants). The Sift and Polyphen Prediction by VEP showed that 100% were NA. Predicted ACMG Outcome by VEP showed that 100% Likely benign, (Table 4 and Figs. 1&2).

In a prior study on this subject, a tiny subset of human HCC patients did not have any BRAF mutations. Missense mutations made up 22.2% (2/9) of SNVs, while nonsense mutations made up 44.5% (4/9). A prior work investigated the MAPK/ERK pathway by means of an NGS panel and a copy-number array. The only recurring missense variation found in their group was a MAPK1 activating mutation, which happened twice. The classic BRAF-activating mutation was also detected in one tumor Haines *et al*. (2019).

Further study documented that among the 77 cases with RAF1 aberrations, 25 cases (32.5%) exhibited RAF1 copy number variants (CNVs) in their tumor samples. In addition, there are

differences in BRAF SNVs according to the type of cancer (HCC 2.3%, Bladder cancer 9.4%, and pancreatic cancer 4.5%) Lim *et al*. (2023).

Of the patients in the Chinese group, 35 cases had 39 different *BRAF* mutations. There were five different kinds of *BRAF* mutations in class 2, including four fusion mutations and one missense mutation. We identified six missenses in the individuals who had a class 3 *BRAF* mutation. In addition, eleven *BRAF* mutations were deemed undescribed. There was one frameshift mutation and one *BRAF* amplification mutation; six were missense kinds Huang *et al*. (2024)

Furthermore, it cannot be considered that distinct etiological variables may contribute to the development of HCC in various populations, which may lead to various methods of transformation. Clinicopathologic characteristics and *BRAF* mutations were significantly associated with (age, family history, HCV infection, ascites, portal vein invasion, and metastasis Tannapfel *et al*. (2003).

SUMMARY

Limitations: Found no evidence of portal vein invasion but did associate *BRAF* mutation to hypertension. Larger tumors tend to have more *BRAF* mutations. More research, including whole exome sequencing, is required to provide a complete explanation for the genetic changes seen in HCC.

The use of NGS led to the discovery of several unique gene mutations in HCC, including both confirmed and disproven mutations. The origin and progression of HCC are best understood because of these findings, which offer new views. Larger patient cohorts are required to fully comprehend *BRAF* genetic alteration and its impact on the development of HCC.

REFERENCES

Becker A. K., Tso D. K., Harris A. C., Malfair D. and Chang, S. D. (2014). extrahepatic metastases of hepatocellular carcinoma: a spectrum of imaging findings. can assoc radiol j., 65: 60-6.

Braunwarth E., Rumpf B., Primavesi F., Pereyra D., Hochleitner M., Göbel G., Gasteiger S., Gehwolf P., Öfner D., Starlinger P. and Stättner S. (2020). sex differences in disease presentation, surgical and oncological outcome of liver resection for primary and metastatic liver tumors-a retrospective multicenter study. Plos One, 15: 43-53.

Caruso S., Calderaro J., Letouzé E., Nault J. C., Couchy G., Boulai A., Luciani A., Zafrani E. S., Bioulac-Sage P., Seror O., Imbeaud S. and Zucman-Rossi J. (2017). germline and somatic dicer1 mutations in familial and sporadic liver tumors. J. Hepatol, 66: 734-42.

Colombino M., Sperlongano P., Izzo F., Tatangelo F., Botti G., Lombardi A., Accardo M., Tarantino L., Sordelli I., Agresti M., Abbruzzese A., Caraglia M. and Palmieri G. (2012). braf and pik3ca genes are somatically mutated in hepatocellular carcinoma among patients from south italy. Cell Death and Disease, 3: e259-e259.

Gnoni A., Licchetta A., Memeo R., Argentiero A., Solimando A. G., Longo V., Delcuratolo S. and Brunetti O. (2019). role of braf in hepatocellular carcinoma: a rationale for future targeted cancer therapies. *Medicina (kaunas)*, 55: 15-32.

Haines K., Sarabia S. F., Alvarez K. R., Tomlinson G., Vasudevan S. A., Heczey A. A., Roy A., Finegold M. J., Parsons D. W. and Plon S. E. (2019). characterization of pediatric hepatocellular carcinoma reveals genomic heterogeneity and diverse signaling pathway activation. Pediatric Blood and Cancer, 66: e27745.

Hassan-Kadle M. A., Osman M. M., Keles E., Eker H. H., Baydili K. N., Ahmed H. M. and Osman A. A. (2022). clinical characteristics of patients with hepatocellular carcinoma: a single-center 3-year experience from somalia. int. J. Hepatol, 20: 33-70.

Hsu C.-Y., Lee, Y.-H., Huang Y.-H., Hsia C.-Y., Su C.-W., Lin H.-C., Lee R.-C., Chiou Y.-Y., Lee F.-Y., Huo T.-I. and Lee S.-D. (2013). ascites in patients with hepatocellular carcinoma: prevalence, associated factors, prognostic impact, and staging strategy. Hepatology International, 7: 188-98.

Huang Y., Jia W., Zhao G., Zhao Y., Zhang S., Li Z. and Wu G. (2024). clinical features and mutation analysis of class 1/2/3 braf mutation colorectal cancer. Chinese Clinical Oncology, cco-23.

Li C. L., Lin Y. K., Chen H. A., Huang C. Y., Huang M. T. and Chang Y. J. (2019). smoking as an independent risk factor for hepatocellular carcinoma due to the α7-nachr modulating the jak2/stat3 signaling axis. J. Clin Med, 8: 45-63.

Li X., Wang X. and Gao P. (2017). diabetes mellitus and risk of hepatocellular carcinoma. Biomed Res. Int., 20: 52-68.

Lim S. H., Jung J., Hong J. Y., Kim S. T., Park S. H., Park J. O., Kim K.-M. and Lee J. (2023). prevalence of raf1 aberrations in metastatic cancer patients: Real-World Data. Biomedicines, 11: 3264.

Loomba R., Liu J., Yang H. I., Lee M. H., Lu S. N., Wang L. Y., Iloeje U. H., You S. L., Brenner D. and Chen C.

J. (2013). synergistic effects of family history of hepatocellular carcinoma and hepatitis b virus infection on risk for incident hepatocellular carcinoma. Clin Gastroenterol Hepatol, 11: 1636-45.

Lopez-Lopez V., Gomez Ruiz A., Lopez-Conesa A., Brusadin R., Cayuela V., Caballero-Illanes A., Torres M. and Robles Campos R. (2020). effects of primary hypertension treatment in the oncological outcomes of hepatocellular carcinoma. Ann. Transl Med, 8: 84-93.

Mak D. and Kramvis A.(2021). epidemiology and aetiology of hepatocellular carcinoma in sub-saharan africa. Hepatoma Res., 7: 26-33.

Mohamed A. A., Elbedewy T. A., El-Serafy M., El-Toukhy N., Ahmed W. and Ali El Din Z. (2015). hepatitis c virus: a global view. World J. Hepatol, 7: 2676-80.

Pope B. J., Clendenning M., Rosty C., Mahmood K., Georgeson P., Joo J. E., Walker R., Hutchinson R. A., Jayasekara H., Joseland S., Como J., Preston S., Spurdle A. B., Macrae F. A., Win A. K., Hopper J. L., Jenkins M. A., Winship I. M. and Buchanan D. D. (2021). germline and tumor sequencing as a diagnostic tool to resolve suspected lynch syndrome. J. Mol. Diagn, 23: 358-71.

Ramadan A., El Ebidy G., Elzaafarany M., Galal A., Ibrahem M. and Ali D. M. (2021). characterization of hepatocellular carcinoma in mansoura university hospitals: a case-control study of risk factors. Medical Journal of Viral Hepatitis, 6: 38-45.

Rashed W. M., Kandeil M. A. M., Mahmoud M. O. and Ezzat S. (2020). hepatocellular carcinoma (hcc) in egypt: a comprehensive overview. J. Egypt Natl Canc Inst, 32: 52-60.

Rich N. E., Murphy C. C., Yopp A. C., Tiro J., Marrero J. A. and Singal A. G. (2020). sex disparities in presentation and prognosis of 1110 patients with hepatocellular carcinoma. Aliment Pharmacol Ther, 52: 701-9.

Russo F. P., Zanetto A., Pinto E., Battistella S., Penzo, B., Burra P. and Farinati F. (2022). hepatocellular carcinoma in chronic viral hepatitis: where do we stand? Int. J. Mol. Sci., 23: 12-52.

Shehta A., Farouk A., Elghawalby A. N., Elshobary M., Aboelenin A., Fouad, A. and Ali M. A. (2021). outcomes of hepatic resection for hepatocellular carcinoma associated with portal vein

invasion. Journal of Surgical Research, 26: 269-83.

Tannapfel A., Sommerer F., Benicke M., Katalinic A., Uhlmann D., Witzigmann H., Hauss J. and Wittekind C. (2003). mutations of the BRAF gene in cholangiocarcinoma but not in hepatocellular carcinoma. Gut, 52: 706-12.

Zhang J., Chen G., Zhang P., Zhang J., Li X., Gan D., Cao X., Han M., Du H. and Ye Y. (2020). the threshold of alpha-fetoprotein (afp) for the diagnosis of hepatocellular carcinoma: a systematic review and meta-analysis. Plos One, 15: 28-85.

Zhao S., Wang M., Yang Z., Tan K., Zheng D., Du X. and Liu L. (2020). comparison between child-pugh score and albumin-bilirubin grade in the prognosis of patients with hcc after liver resection using time-dependent roc. Ann. Transl Med, 8: 53-9.

Table (1): *BRAF* mutation in HCC cases according to demographic and clinical characteristics.

Variables		Mutant type (n=10)		Wild type (n=11)		Total N=21		OR (95% CI)	*P*-value
		No.	%	No.	%	No.	%		
Sex	**Male**	7	%70	11	100%	18	85.7 %	0.70 (0.195-2.511)	0.584
	Female	3	%30	0	0.0%	3	14.3 %		
Smoking	**Yes**	1	10%	1	9.1%	2	9.5%		
	No	8	%80	6	54.5%	14	66.6%	1.467 (0.376-5.723)	0.581
	Ex. smoker	1	%10	4	36.4%	5	23.8%		
Bilharzia	**Yes**	6	%60	7	63.6%	13	61.9%	0.943 (0.236-3.772)	0.934
	No	4	%40	4	36.4%	8	38.1%		
Hepatic encephalopathy	**Yes**	0	0.0%	0	0.0%	0	0%	1.00 (0.298-3.357)	1.00
	No	10	100%	11	100%	21	100 %		
Family history	**Yes**	1	%10	3	27.3%	4	19.1 %	1.238 (0.344-4.454)	0.744
	No	9	%90	8	72.7%	17	80.9 %		

Viral infection	**HCV**	10	%100	9	81.8%	19	90.5 %	1.222 (0.353-4.235)	0.752
	HBV	0	.0%0	1	9.1%	1	4.7%	0.365 (0.013-9.979)	0.551
	NBNC	0	0.0%	1	9.1%	1	4.7 %	0.365 (0.013-9.979)	0.551
Comorbidities	**DM**	2	20%	5	45.5%	7	33.3 %	0.44 (0.069-2.798)	0.384
	HTN	2	20%	1	9.1%	3	14.3 %	2.20 (0.172-28.139)	0.544

Data are presented as frequency (%) or mean ± SD. HCC: Hepatocellular carcinoma, HCV: hepatitis C virus, HBV: hepatitis B virus, DM: diabetes mellitus. HTN: hypertension. *Significant. P value <0.05, CI: confidence interval, OR: odds ratio.

Table (2): Clinicopathological features of HCC patients according to *BRAF* gene mutation.

Variables	Mutant type (n = 10)		Wild type (n=11)		Total		*P*- value
	No.	%	No.	%	No.	%	
	Ascites						
No	9	90%	8	72.7%	17	90.0 %	0.744
Minimal	0	0.0%	1	9.1%	1	4.7 %	
Moderate	1	10%	2	18.2%	3	14.3 %	
	Portal Vein Invasion						
Negative	9	90%	9	81.8%	18	85.7 %	0.882
Positive	1	10%	2	18.2%	3	14.3 %	
	LN Metastasis						
Negative	10	100%	8	72.7%	18	85.7 %	0.622
Positive	0	0.0%	3	27.3%	3	14.3 %	
	Lung Metastasis						
Negative	9	90%	9	81.8%	18	85.7 %	0.882
Positive	1	10%	2	18.2%	3	14.3 %	
	Child PUGH class						

Table (2)Cont'							
A	8	80%	8	72.7%	16	76.2 %	0.195
B	0	0.0%	3	27.3%	3	14.3 %	
C	2	20%	0	0.0%	2	9.5 %	
	CT radiological findings						
	Tumor number						
Single	5	50%	4	36.4%	9	42.9 %	0.691
Multiple	5	50%	7	63.6%	12	57.1 %	
	Tumor Size						
Small (<3 cm)	3	30%	0	0.0%	3	14.3 %	0.691
Medium (3 - 5 cm)	0	0.0%	1	9.1%	1	4.7 %	
Large (>5 cm)	7	70%	10	90.9%	17	90.0 %	
	BCLC						
A	4	40%	3	27.3%	7	33.3%	0.085
B	4	40%	1	9.1%	5	23.8%	
C	0	0.0%	7	63.6%	7	33.3%	
D	2	20%	0	0.0%	2	9.5 %	

Data are presented as frequency (%) or mean ± SD. PVI: portal vein invasion. BCLC: Barcelona clinic liver cancer. *Significant. P value <0.05.

Table (3): AFP (ng/mL) level in the HCC population.

HCC	(n = 21)
AFP (ng/mL)	
Min. – Max.	4.9– 42443
Mean ± SD.	2417.07± 9230.79
Median	42.05 (18.4–107.5)

Table (4): Summary of *BRAF* gene variation in HCC detected by targeted sequencing.

Locus	Types	Variant frequency	genes	Amino Acid Change	Coding
chr7:140453102	CNV	0.44	*BRAF,EZH2*	0	0
chr7:140453106	SNV	0.04	*BRAF*	p.Phe610Ser	c.1829T>C
chr7:140453111	SNV	0.04	*BRAF*	p.His608Gln	c.1824T>G
chr7:140453147	SNV	0.04	*BRAF*	#N/A	#N/A
chr7:140453183	SNV	0.04	*BRAF*	#N/A	#N/A
chr7:140453207	SNV	0.04	*BRAF*	p.?	c.1742-14T>C
chr7:140453217	SNV	0.04	*BRAF*	p.?	c.1742-24T>C
chr7:140481398	INDEL	0.04	*BRAF*	p.Val471dup	c.1409_1410insGGT
chr7:140481479	SNV	0.04	*BRAF*	#N/A	#N/A
chr7:140481504	SNV,INDEL	0.12	*BRAF*	p.?	c.1315-12_1315-11insGCAGGC

Chr: Chromosome, SNV: single nucleotide variant, CNV: copy number variant, INDEL: insertions/deletion variants, N/A: not applicable

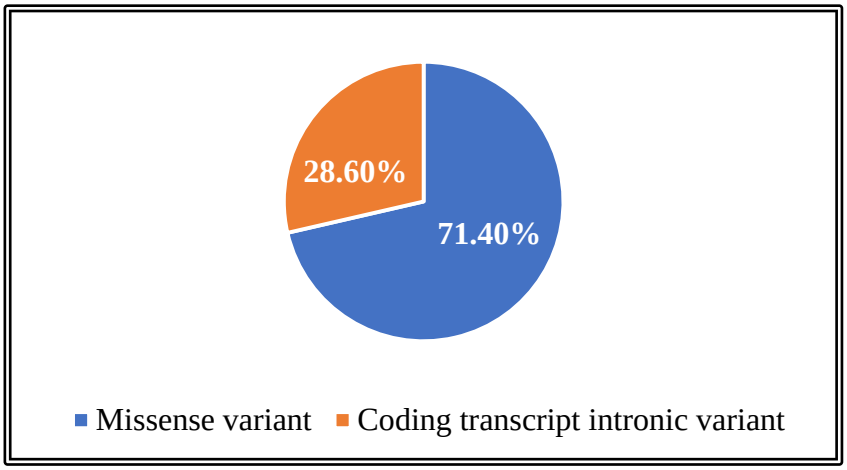

Fig. (1): Percentage of Variant effect of SNVs

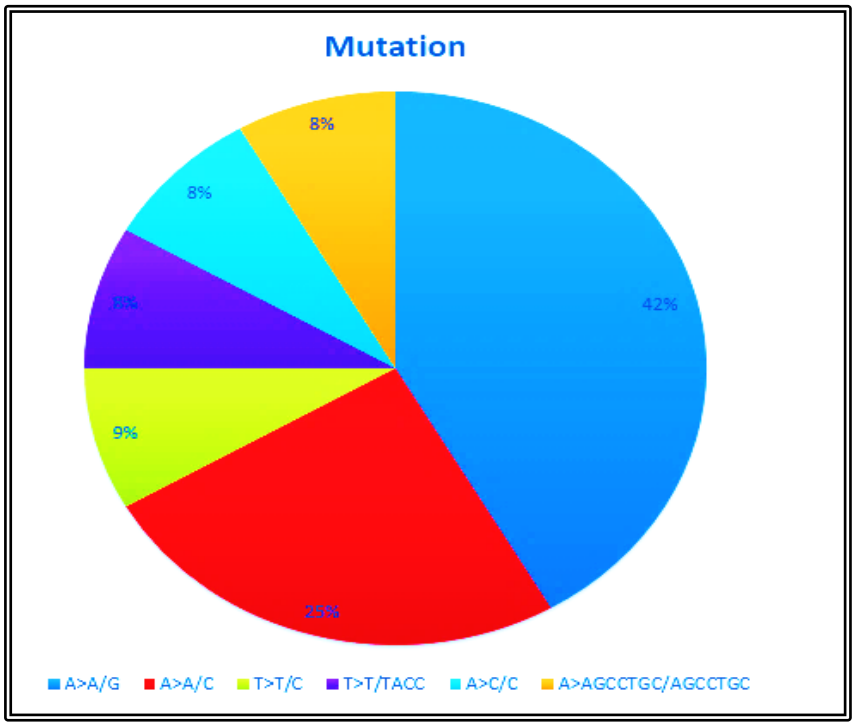

Fig. (2): Summary of *BRAF* mutations in HCC patients.

GENE SEQUENCING OF *EGFR* IN HEPATOCELLULAR CARCINOMA PATIENTS

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Keywords: *EGFR* gene, Hepatocellular carcinoma, Next generation sequencing (NGS), DNA extraction, Genetic alternations.

Hepatocellular carcinoma HCC is the fourth most prevalent cause of cancer-related mortality globally. Approximately 80-90% of HCC cases are associated with cirrhosis, which can be attributed to a chronic infection of the hepatitis B or hepatitis C virus (HCV). Many individuals with HCC are not candidates for potentially curative therapy such as surgical resection and transplantation due to their advanced stage of the disease Russo *et al*. (2022). In Egypt, it is the fourth most common cancer. Multiple examinations conducted in hospitals have shown an increase in the incidence of HCC Rashed *et al*. (2020).

The *EGFR* system regulates cell proliferation, survival, and migration. Its aberrant activity has been associated with the onset and progression of a variety of malignancies, including HCC. Overexpression of *EGFR* and its ligands has been associated with aggressive liver cancer with a low survival rate Berasain *et al*. (2011). *EGFR* overexpression is common in HCC, suggesting that it may play an important role in HCC etiology and treatment. Furthermore, *EGFR*

activation has been proposed as a potential predictor of primary resistance in HCC cells Guardiola *et al*. (2019). The objective of the present investigation was to examine the relationship between *EGFR* and the progression of HCC in HCC patients through the utilization of NGS technology.

MATERIALS AND METHODS

a. Study design and subjects

This study was conducted on a group of twenty-one patients who were suffering from HCC and were referred to the National Liver Institute Hospital at Menoufia University, Egypt. The study was approved and took place between January and November 2020. The patients, consisting of 18 males and 3 females with an average age of 62, had human HCC and were attending the oncology clinic at the National Liver Institute. The study only included patients suffering from HCC, and those with other types of cancers were excluded. The patient's genomes were analyzed against three healthy individuals who did not have any tumors. Each patient had a comprehensive evaluation, which included a clinical examination, tumor staging, thorough laboratory testing (such as coagulation profile, liver enzymes, renal function tests, and a complete blood count), and a chest X-ray. Menoufia University's Ethics Committee, namely the National Liver Institute, approved the project.

b. Sample collection and cell-free DNA extraction

Full blood samples were used for genomic DNA extraction after collecting 1-3 mL of peripheral blood in EDTA-containing tubes. A temperature of -80ºC was subsequently used to store the plasma. The QIAamp® DSP Virus spin kit Version 1 (QIAGEN, Hilden, Germany) was used to treat the plasma to extract DNA from circulating cells.

c. Next-generation sequencing

Genomic DNA was extracted using the Gene JET Genomic DNA Purification Kit (Thermoscientific, Cat#K0721). For library preparation, a total of 10 nanograms (ng) of DNA was amplified using the Ion AmpliSeqTM HiFi Master Mix and the Ion AmpliSeqTM Cancer Hotspot Panel (version 2; Thermo Fisher Scientific,Inc.).

The ion library TaqMan® Quantitation Kit (Thermo Fisher Scientific, Inc.) was used for qPCR quantification of the library according to the manufacturer's instructions. The templates were prepared and amplified using the Ion OneTouchTM2 technology. Thermo Fisher Scientific, Inc.'s Ionosphere quality control kit was used to ensure that 10% to 30% of the manufactured ISPs were template positive. Following enrichment, the template ISPs were transferred to Ion 316TM chips. The IonPGMTM Sequencing Hi-Q view kit v2 and PGMTM (Life Technologies) were

used for sequencing, following the manufacturer's instructions.

d Bioinformatics data analysis

The analysis of normal and malignant samples was conducted using the ion amplifier custom panel approach. The data was compared to Human Genome Version 19 (hg19) using Thermo Fisher's Ion reporter server 5.10, with the default plugin parameters employed. This comparison was conducted using Torrent Suite (version 3.6.2; Thermo Fisher Scientific, Inc.). The Coverage Analysis plugin (version 3.6; Thermo Fisher Scientific, Inc.) was employed in this study. The quality thresholds, average base coverage, allele frequency, and general uniformity were set at >20, >500x reads, >10%, and >80%, respectively. Mutations were discovered using the Variant Caller plugin (version 3.6; Thermo Fisher Scientific, Inc.). Subsequently, the validation of each mutation was conducted utilizing the Integrated Genome Viewer (IGV) provided by the Broad Institute (www.broadinstitute.org).

Statistical analysis

Using SPSS version 28, which was created in the Illinois city of Chicago, USA, the statistical assessment was carried out. Categorical variables were represented by frequencies and percentages, whereas continuous variables were represented by means, standard deviations, or medians (IQR). For continuous variables, we made use of the Mann-Whitney U test; for categorical variables, we used the Chi-square test to determine statistical significance. The threshold for statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

There were 18 men (85.7% of the total) and 3 females (14.3%) in the research. Patients' ages were used to classify them into two groups: one for those 60 and above (61.9% of the total) and another for those 60 and younger (38.1%). The average age in group 1 was 62.19 ±8.85 years, while the median age was 63 years. We found that thirteen patients (61.9%) tested positive for bilharzia antibodies, nineteen patients (90.5%) tested positive for HCV, and one patient (4.75%) tested positive for HBV. Comorbidities are shown in (Table 1), with 7 cases (33.3%) having diabetes mellitus (DM) and 3 cases (14.3%) having hypertension (HTN). This agreed with Ikeda *et al*., (2018) who that found out of the 14 patients diagnosed with HCC, 85.7% were men with a median age of 62 years. Previous studies have linked the male predominance in HCC to increased exposure to risk factors, androgens (AR), and estrogens (ER) Zhang *et al*. (2020). As opposed to Tanaka *et al*. (2010), those who claimed that the higher prevalence of adenocarcinoma in females is reflected in the female predominance in HCC.

There was no statistically significant correlation between smoking and HCC. The association between HCC and tobacco exposure was further

supported by the finding of two out of the eight molecular markers that are linked to HCC. According to reports, smoking has several harmful consequences on the liver, such as liver carcinogens Li *et al*. (2019).

Based on the findings of this investigation, 61.9% of the 21 patients with HCC tested positive for bilharzia antibodies. These findings align with the research conducted by Ramadan *et al*. who found that Schistosoma antibodies of HCC Egyptian patients were (67.7%) of all 220 HCC patients Ramadan *et al*. (2021). The risk of developing primary liver cancer was shown to be up to 50% higher in people who smoked compared to those who did not smoke. It was also determined that 64 percent of Egyptians diagnosed with HCC smoke. In Egypt, excessive smoking is a major risk factor for non-B or non-C HCC, according to Abou El Azm *et al*. (2014). According to another research, smoking is a major factor in the development of HCC in Egypt Brozzetti *et al*. (2021). A second Egyptian study found that people who smoked 20 cigarettes daily for over 29 years had a higher chance of developing HCC Moustafa *et al*. (2009).

According to this study, anti-HCV antibodies were present in 90.5% of all 21 patients, but only 4.75% of total cases had HBV. It was demonstrated that HCV was the predominant cause of HCC in Egypt, and it continues to be the main factor in the development of HCC in Europe, North America, and Japan Brozzetti *et al*. (2021).

The results of this study agreed with those of an earlier one that indicated that 33.5 percent had a history of heavy alcohol consumption, 24.3 percent had viral hepatitis, and 33.5 percent had both diagnoses. Among the participants, 29.9% had diabetes, 37.9% had hypertension, and 35.9% smoked cigarettes Raffetti *et al*. (2015). Among the cohort of 21 patients, there is 19.0% had a familial predisposition to liver cancer. There is no significant correlation between a familial predisposition and HCC. Nevertheless, Caruso *et al*. have observed a significant correlation between a familial background of liver cancer and a heightened susceptibility to the development of HCC characterized by aggressive characteristics Caruso *et al*. (2017).

According to this study, DM incidence was in only 33.3% of all HCC cases with the same line of the baseline clinical characteristics of the previous study of the whole study population that indicated that DM incidence was present in (24.1%) among all HCC patients Ramadan *et al*. (2021). Second research found that having type 2 diabetes doubles or even triples the probability of having HC Ziada *et al*. (2016).

The rise in HCC identification in Egypt may be attributed to the implementation of a comprehensive screening program aimed at finding and treating HCV. Several people were diagnosed and treated for HCC as a result of this initiative. Other researchers conducted a study that revealed that 75%

of hepatocellular carcinoma (HCC) cases originated from rural regions in Egypt. Additionally, 45.7% of the affected individuals fell within the age range of 51-60 years Zhao *et al*. (2020).

This study found that ascites were identified in (19.0%) of patients (3 minimal and 1 moderate) of all 21 patients at the time of diagnosis. Three (14.3%) HCC patients with (PVI) were significantly correlated with HCC (P = 0.01). The most common metastatic sites were the lungs (14.3%) and the lymph nodes (14.3%). A previous study reported that brain metastasis was (2%), peritoneum (11%), adrenal glands (11%), bone (28%), local lymph nodes (53%), and lung (55%) which were the most frequent extrahepatic HCC metastatic locations to their frequencies Zhao *et al*. (2020).

Extrahepatic metastasis is a sign of advanced HCC, according to clinical standards. The classic Child-Pugh rating system has been the most popular way to evaluate liver function and determine the effectiveness of treatments for many years Zhao *et al*. (2020). Various staging systems, including the BCLC staging system, have been proposed in recent years. The Child-Pugh score, tumor burden, and patient performance status are only a few factors the BCLC staging system considers Hsu *et al*. (2013).

According to this current study, 81% of the cases had tumors measuring more than 5cm in diameter based on CT scans of the population. Among these cases, 57.1% had multiple lesions lesion, and 42.9% had a single lesion. According to the BCLC staging, stages A and C had a higher prevalence rate of 33.35% apiece. The results of the Child-Pugh score indicate that Child's A accounted for 76.2% of the total, followed by Child's B at 14.3% and Child's C at 9.5% (Table 2). The aforementioned results align with the data reported by Hassan-Kadle *et al*. (2022)., wherein it was seen that 73.6% of the patients fell into the Child-Pugh classification, with 17.2% classified as Child's A and 9.0% classified as Child's C Furthermore, the aforementioned findings were consistent with the research which reported that 64.3% of patients exhibited Child-Pugh B or C cirrhosisIkeda *et al*. (2018). According to another research, 162 patients (73.6%) had portal veins, with 104 patients (47.3%) having multiple lesions in the right lobe. Additionally, 180 patients (81.8%) had cirrhosis, 104 patients (47.3%) had BCLC stage D, and 105 patients (47.7%) were classified as child B %) Ramadan *et al*. (2021).

Based on the demographic and clinical data at hand, it is evident that the *EGFR*-mutated group had a notably greater representation of male participants, non-smokers, in contrast to the Wild-Type group, as well as those whose tumors were either mildly or fairly differentiated. Based on our research, it appears that the higher frequency of *EGFR* mutation in males may be linked to a higher incidence of HCC. Our findings indicate that the higher prevalence of *EGFR* mutations in

males is indicative of a greater occurrence of HCC. The correlation between patients and *EGFR* mutations was significantly more likely to be male (84.2%). *EGFR* gene mutations were also significantly more likely to be non-smoker (73.7%) and without family history (78.9%). Out of all muted patients significantly more likely to have HCV (89.5%), 78.9% without ascites, 15.8% had positive PVI, 5.3% had metastasis in lung and lymph node and 15.8% had HTN. The large tumor size (more than 5 cm) was significantly predominant (84.2%). There was a negative correlation between *EGFR* gene mutation in HCC cases and clinical characteristics and clinic-pathological features. as shown in (Tables 3&4).

According to a study conducted by Lin *et al.* (2020), there were more women and people without smoking in the *EGFR* mutant group, and there were more people with tumors that were well- or moderately differentiated compared to the wild-type group. These findings are in direct opposition to prior research.

The study revealed that there was no significant correlation between *EGFR* mutation status and demographic and clinical features, including age, degree of differentiation, clinical stages, tumor size, viral infection, and other pathological differentiation ($P > 0.05$). The research that demonstrated that there is no correlation between age and pathogenic differentiation with *EGFR* mutations Zhou *et al.* (2019). On the other hand, another study determined that the overall number of poor and moderate differentiation was lower in *EGFR* mutation-harboring SqCLC patients compared to wild-type patients Zhang and Junling (2016).

All patients underwent *EGFR* gene sequencing, and mutations were identified in 19/21 (90.5%) of the samples. All detected mutations were 55 variants (Table 5). There were 39/55 (70.9%) single nucleotide variants (SNVs), 3/55 (5.5%) multi-nucleotide variants (MNVs), 8/55 (14.5%), copy number variants (CNVs) and 5/55 (9.1%) insertions/deletion variants (INDELs). The Ensembl Variant Effect Predictor (VEP) was utilized to further interpret and filter genomic variants. It predicts the molecular consequences of variants using the Ensembl/GENCODE or RefSeq gene sets. Out of the SNVs and INDELs, 26 out of 44 (59.1%) were found to be somatic mutations, while 18 out of 44 (40.9%) were germline mutations. There were also both novel and existing mutations present, with 26 out of 44 (59.1%) being novel and 18 out of 44 (40.9%) being existing mutations. The Variant effect showed that there were 14 (31.8%) missense variants, 6 (13.6%) synonymous variants, 18 (40.9%) coding transcript intronic variants, 1 (2.3%) stop-gained variants, 4 (9.1%) splice region variants and 1 (2.3%) splice donor variants. Predicted ACMG Outcome by VEP showed that 3 (6.8%) were Likely pathogenic, 13 (29.6%) Uncertain significance, 2 (4.5%) Benign, 26 (59.1%) Likely benign (figs. 1&2&3).

A previous study aimed to assess the frequency of concurrent genetic changes in a cohort of 54 individuals diagnosed with advanced lung cancer. To achieve this, a series of gene assays were conducted, encompassing a range of somatic genetic alterations Deng *et al*. (2019). Specifically, the study focused on 24 patients with *EGFR* mutations and 30 patients with *EGFR* mutations who were in the advanced stage of lung cancer (stage IIIB or IV). NGS was used to test copy number variants (CNV), inframe_indel, fusions, frameshift, missense, splicing, and stop acquired in 422 clinically important cancer genes. The findings of this current study are consistent with our research outcomes. Among the mutant type of *EGFR*, there were 4 out of 24 CNVs, 8 out of 24 inframe indel mutations, and 16 out of 24 missense mutations. In contrast, the wild-type *EGFR* had less than 2 out of 30 CNVs and no other differences were observed Deng *et al*. (2019).

On the other hand, there was study that evaluated 14 patients with advanced HCC. The calculation was performed to determine the proportion of mutant alleles concerning wild-type alleles. Every individual in the study exhibited at least one somatic mutation, with a median of three mutations per patient (ranging from 1 to 8). The mutant alleles had a median percentage of 0.29%, with a range of 0.1% to 37.77%. A total of 14% of mutations were detected in the *EGFR* gene. Furthermore, a comprehensive analysis was conducted on the complete exons of 29 genes, as well as critical exons specifically identified as having somatic variants in the COSMIC dataset. The purpose of this investigation was to detect and record SNVs, amplifications of 16 genes' copy numbers, fusions of ALK/RET/ROS1/NTRK1, and *EGFR* insertion/deletion mutations Ikeda *et al*. (2018).

An investigation was carried out on the *EGFR* gene in 100 patients with HCC and 102 patients with nasopharyngeal carcinoma, with a specific focus on exons 18-21. A silent exonic mutation in exon 20, 2361G > A (Q787Q), was identified in 32% of samples from HCC and 41% of samples from nasopharyngeal cancer, according to the study. In exon 20, a silent exonic mutation, specifically 2457G > A (V819V), was identified and was observed in 3% of the samples of nasopharyngeal cancer. There were no further mutations detected in exons 18 to 21 in the samples of hepatocellular and nasopharyngeal cancer. Eight intronic mutations were identified Lee *et al*., (2006).

SUMMARY

Limitations: found no evidence of portal vein invasion but did associate *EGFR* mutation with hypertension. Larger tumors tend to have more *EGFR* mutations. Further investigations, such as whole exome sequencing, are required to comprehensively elucidate the genetic modifications in HCC NGS facilitated the identification of numerous distinct gene variants in HCC, encompassing both validated and invalidated mutations. The

understanding of the origin and course of hepatocellular carcinoma (HCC) is enhanced by these results, which provide novel perspectives. To completely understand the impact of *EGFR* genetic change on the development of HCC, it is necessary to have larger patient cohorts.

REFERENCES

Abou El Azm A. R., Yousef M., Mansour N., Awad A., El Dardiry S. and Abdel Aziz I., (2014). New insights on non-B non-C hepatocellular carcinoma in mid Delta Region, Egypt. Journal of Gastrointestinal Cancer, 45: 276-283.

Berasain C., Ujue Latasa M., Urtasun R., Goñi S., Elizalde M., Garcia-Irigoyen O., Azcona M., Prieto J. and Ávila M. A., (2011). Epidermal Growth Factor Receptor *(EGFR)* Crosstalks in Liver Cancer. Cancers, 3: 2444-2461.

Brozzetti S., Tancredi M., Bini S., De Lucia C., Antimi J., D'alterio C., De Sanctis G. M., Furlan C., Malpassuti V. C. and Lucatelli P., (2021). HCC in the era of direct-acting antiviral agents (DAAs): surgical and other curative or palliative strategies in the elderly. Cancers, 13: 3025.

Caruso S., Calderaro J., Letouzé E., Nault J.-C., Couchy G., Boulai A., Luciani A., Zafrani E.-S., Bioulac-Sage P. and Seror O., (2017). Germline and somatic DICER1 mutations in familial and sporadic liver tumors. Journal of Hepatology, 66: 734-742.

Deng L.-L., Gao G., Deng H.-B., Wang F., Wang Z.-H. and Yang Y., (2019). Co-occurring genetic alterations predict distant metastasis and poor efficacy of first-line *EGFR*-TKIs in *EGFR*-mutant NSCLC. Journal of Cancer Research and Clinical Oncology, 145: 2613-2624.

Guardiola S., Varese M., Sanchez-Navarro M. and Giralt E., (2019). A third shot at *EGFR*: new opportunities in cancer therapy. Trends in Pharmacological Sciences, 40: 941-955.

Hassan-Kadle M. A., Osman M. M., Keles E., Eker H. H., Baydili K. N., Ahmed H. M. and Osman A. A., (2022). Clinical characteristics of patients with hepatocellular carcinoma: a single-center 3-year experience from Somalia. International Journal of Hepatology, 2022.

Hsu C.-Y., Lee Y.-H., Huang Y.-H., Hsia C.-Y., Su C.-W., Lin H.-C., Lee R.-C., Chiou Y.-Y., Lee F.-Y. and Huo T.-I., (2013). Ascites in patients with hepatocellular carcinoma: prevalence, associated factors, prognostic impact, and staging strategy. Hepatology international, 7: 188-198.

Ikeda S., Tsigelny I. F., Skjevik Å. A., Kono Y., Mendler M., Kuo A., Sicklick J. K., Heestand G., Banks K. C. and Talasaz A., (2018). Next-generation sequencing of circulating tumor DNA reveals frequent alterations in advanced hepatocellular carcinoma. The oncologist, 23: 586-593.

Lee S.-C., Lim S.-G., Soo R., Hsieh W.-S., Guo J.-Y., Putti T., Tao Q., Soong R. and Goh B.-C., (2006). Lack of somatic mutations in *EGFR* tyrosine kinase domain in hepatocellular and nasopharyngeal carcinoma. Pharmacogenetics and Genomics, 16: 73-74.

Li C.-L., Lin Y.-K., Chen H.-A., Huang C.-Y., Huang M.-T. and Chang Y.-J., (2019). Smoking as an independent risk factor for hepatocellular carcinoma due to the α7-nachr modulating the JAK2/STAT3 signaling axis. Journal of clinical medicine, 8: 1391.

Lin C.-H., Yang P.-J., Lin S.-H., Yeh K.-T., Tsao T. C.-Y., Chen Y.-E., Lin S.-H. and Yang S.-F., (2020). Association between *EGFR* gene mutation and antioxidant gene polymorphism of non-small-cell lung cancer. Diagnostics, 10: 692.

Moustafa E. F. A., Galal G. M., Aly A. and Hemeyda K., (2009). (127) Smoking and the risk of hepatocellular carcinoma among Egyptian patients. A preliminary case-control study. Arab Journal of Gastroenterology, 2: AB54.

Raffetti E., Portolani N., Molfino S., Baiocchi G. L., Limina R. M., Caccamo G., Lamera R., Donato F. and Group B. H. S., (2015). Role of aetiology, diabetes, tobacco smoking and hypertension in hepatocellular carcinoma survival. Digestive and Liver Disease, 47: 950-956.

Ramadan A., El Ebidy G., Elzaafarany M., Galal A., Ibrahem M. and Ali D. M., (2021). Characterization of hepatocellular carcinoma in Mansoura university Hospitals: A case-control study of risk factors. Medical Journal of Viral Hepatitis, 6: 38-45.

Rashed W. M., Kandeil M. A. M., Mahmoud M. O. and Ezzat S., (2020). Hepatocellular Carcinoma (HCC) in Egypt: A comprehensive overview. Journal of the Egyptian National Cancer Institute, 32: 1-11.

Russo F. P., Zanetto A., Pinto E., Battistella S., Penzo B., Burra P. and Farinati F., (2022). Hepatocellular carcinoma in chronic viral hepatitis: where do we stand? International journal of molecular sciences, 23: 500.

Tanaka T., Matsuoka M., Sutani A., Gemma A., Maemondo M., Inoue A., Okinaga S., Nagashima M., Oizumi S. and Uematsu K., (2010). Frequency of and variables associated with the *EGFR* mutation and its subtypes. International journal of cancer, 126: 651-655.

Zhang J., Chen G., Zhang P., Zhang J., Li X., Gan D. N., Cao X., Han M., Du, H. and Ye Y. A., (2020). The threshold of alpha-fetoprotein (AFP) for the diagnosis of hepatocellular carcinoma: A systematic review and meta-analysis. *PLoS One,* 15: e0228857.

Zhang T. and Junling L., (2016). Driven gene in patients with lung squamous cell carcinoma: analysis of clinicopathologic characteristics and prognosis. Zhongguo Fei Ai Za Zhi, 19.

Zhao S., Wang M., Yang Z., Tan K., Zheng, D., Du X. and Liu L., (2020). Comparison between Child-Pugh score and Albumin-Bilirubin grade in the prognosis of patients with HCC after liver resection using time-dependent ROC. Annals of translational medicine, 8.

Zhou S., Wang H., Jiang W. and Yu Q., (2019). Clinicopathological characteristics and *EGFR*-TKIs efficacies in lung squamous cell carcinoma patients harboring an *EGFR* sensitizing mutation. OncoTargets and therapy, 8863-8871.

Ziada D. H., El Sadany S., Soliman H., Abd-Elsalam S., Salama M., Hawash N., Selim A., Hamisa M. and Elsabagh H. M., (2016). Prevalence of hepatocellular carcinoma in chronic hepatitis C patients in Mid Delta, Egypt: A single center study. Journal of the Egyptian National Cancer Institute, 28: 257-262.

Table (1): HCC patient characteristics and risk factors.

Variables		HCC (n = 21) No. (%)	OR (95% CI)	P-value
Sex	**Male**	18 (85.7%)	-	-
	Female	3 (14.3%)	-	-
Age (years)		62.19 ±8.85	-	-
Age groups (years)	**<60** **>60**	8 (38.1%) 13 (61.9%)	-	-
Risk factors				
Smoking	**Yes** **Ex. Smoker**	2 (9.5%) 5 (23.8%)	NA	0.213
Bilharzia	**Yes**	13 (61.9%)	1.625 (0.558- 4.730)	0.373
Hepatic encephalopathy	**Yes**	0 (0.0%)	0.023 (0.001- 0.409)	0.01*
Family history	**Yes**	4 (19.0%)	0.235 (0.068- 0.818)	0.023*
Viral infection	**HCV** **HBV** **NBNC**	19 (90.5%) 1 (4.75%) 1 (4.75%)	9.50 (1.96- 46.01) 0.050 (0.006- 0.407) 0.050 (0.006- 0.407)	0.005*
Comorbidities	**DM** **HTN**	7 (33.3%) 3 (14.3%)	0.50 (0.168- 1.488) 0.167 (0.043- 0.652)	0.213 0.01*

Data are presented as frequency (%) or mean ± SD. HCC: Hepatocellular carcinoma, HCV: hepatitis C virus, HBV: hepatitis B virus, DM: diabetes mellitus. HTN: hypertension. *Significant. P value <0.05, CI: confidence interval, OR: odds ratio.

Table (2): Clinicopathological features of HCC patients.

Variables		HCC (n = 21) No. (%)	OR (95% CI)	P- value
Ascites	**No** **Minimal** **Moderate**	17 (81.0%) 3 (14.3%) 1 (4.7%)	NA	0.023*
Portal Vein Invasion	**Negative** **Positive**	18 (85.7%) 3 (14.3%)	6.00 (1.53- 23.47)	0.01*
LN Metastasis	**Negative** **Positive**	18 (85.7%) 3 (14.3%)	6.00 (1.53- 23.47)	0.01*
Lung Metastasis	**Negative** **Positive**	18 (85.7%) 3 (14.3%)	6.00 (1.53- 23.47)	0.01*
Child PUGH class	**A** **B** **C**	16 (76.2%) 3 (14.3%) 2 (9.5%)	NA	0.05*
CT radiological findings				
Tumor number	**Single** **Multiple**	9 (42.9%) 12 (57.1%)	-	-
Tumor Size	**Small (<3 cm)** **Medium (3 - 5 cm)** **Large (>5 cm)**	3 (14.3%) 1 (4.7%) 17 (81.0%)	-	-
BCLC	**A** **B** **C** **D**	7 (33.35%) 5 (23.8%) 7 (33.35%) 2 (9.5%)	-	-

Data are presented as frequency (%) or mean ± SD. BCLC: Barcelona clinic liver cancer. *Significant. P value <0.05.

Table (3): *EGFR* mutation in HCC cases according to demographic and clinical characteristics.

Variables		Mutant type (n=19)		Wild type (n=2)		OR (95% CI)	*P*-value
		No.	%	No.	%		
Sex	**Male**	16	84.2%	2	100%	0.842 (0.106-6.672)	0.871
	Female	3	15.8%	0	0%		
Smoking	**Yes**	2	10.5%	0	0.0%	NA	0.408
	No	14	73.7%	0	0.0%		
	Ex. smoker	3	15.8%	2	100%		
Bilharzia	**Yes**	13	68.4%	0	0.0%	3.462 (0.154- 77.98)	0.435
	No	6	31.6%	2	100%		
Hepatic encephalopathy	**Yes**	0	0.0%	0	0%	1.00 (0.262-3.815)	1.00
	No	19	100.0%	2	100%		
Family history	**Yes**	4	21.1%	0	0.0%	1.154 (0.047-28.44)	0.930
	No	15	78.9%	2	100%		
Viral infection	**HCV**	17	89.5%	2	100%	0.895 (0.113-7.065)	0.916
	HBV	1	5.25%	0	0%	0.385 (0.012- 12.249)	0.588
	NBNC	1	5.25%	0	0.0%	0.385 (0.012- 12.249)	0.588
Comorbidities	**DM**	6	31.6%	1	50%	0.632 (0.048-8.252)	0.726
	HTN	3	15.8%	0	0.0%	0.897 (0.035-22.975)	0.948

Data are presented as frequency (%) or mean ± SD. HCC: Hepatocellular carcinoma, HCV: hepatitis C virus, HBV: hepatitis B virus, DM: diabetes mellitus. HTN: hypertension. *Significant. P value <0.05, CI: confidence interval, OR: odds ratio.

Table (4): *EGFR* mutation in HCC cases according to clinicopathological characteristics.

Variables	Mutant type (n = 19)		Wild type (n=2)		*P*- value
	No.	%	No.	%	
Ascites					
No	15	78.9%	2	100%	0.823
Minimal	3	15.8%	0	0.0%	
Moderate	1	5.3%	0	0.0%	
Portal Vein Invasion					
Negative	16	84.2%	2	100%	0.871
Positive	3	15.8%	0	0.0%	
LN Metastasis					
Negative	18	94.7%	0	0.0%	0.325
Positive	1	5.3%	1	100%	
Lung Metastasis					
Negative	18	94.7%	0	0.0%	0.325
Positive	1	5.3%	1	100%	
Child PUGH class					
A	14	73.7%	2	100%	0.773
B	3	15.8%	0	0.0%	
C	2	10.5%	0	0.0%	

Table (4):Cont’					
CT radiological findings					
Tumor number					
Single	9	47.4%	0	0.0%	0.578
Multiple	10	52.6%	2	100%	
Tumor Size					
Small (<3 cm)	2	10.5%	1	50%	0.682
Medium (3 - 5 cm)	1	5.3%	0	0.0%	
Large (>5 cm)	16	84.2%	1	50%	
BCLC					
A	7	36.9%	0	0.0%	0.684
B	5	26.3%	0	0.0%	
C	5	26.3%	2	100%	
D	2	10.5%	0	0.0%	

Data are presented as frequency (%) or mean ± SD. PVI: portal vein invasion. BCLC: Barcelona clinic liver cancer. *Significant. P value <0.05.

Table (5): Summary of *EGFR* gene variation in HCC detected by targeted sequencing.

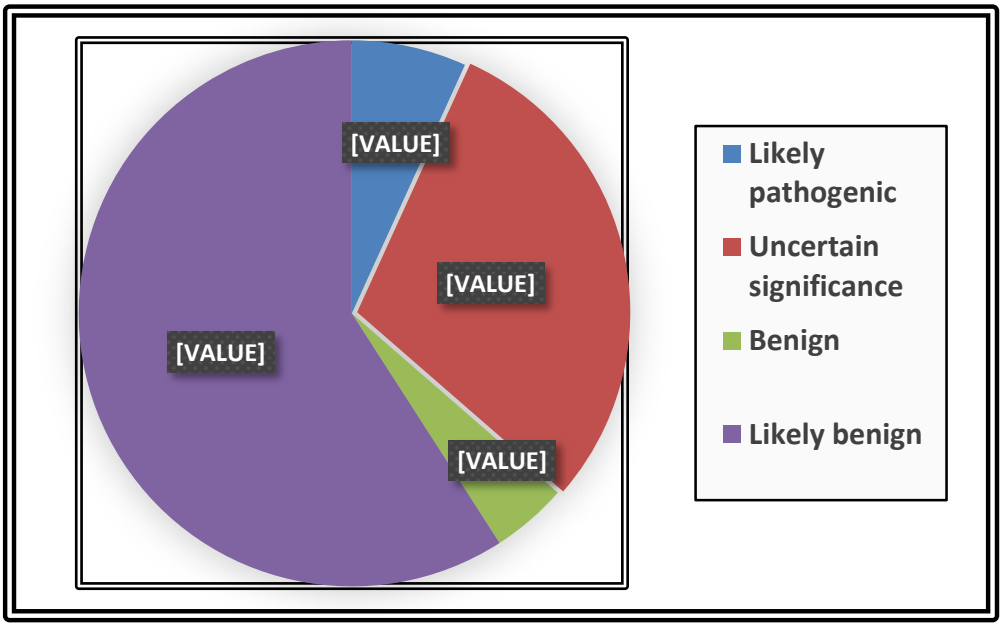
Locus	Types	Variant frequency	Genes	Amino acid change	Coding
chr7:55211044	**CNV**	0.28	*EGFR*-AS1, MET, *EGFR*	0	0
chr7:55211044	**CNV**	0.04	*EGFR-AS1, EGFR*	0	0
chr7:55211044	**CNV**	0.12	*EGFR*	0	0
chr7:55211044	**CNV**	0.08	*EGFR*	0	0
chr7:55211044	**CNV**	0.12	*EGFR*	0	0
chr7:55211097	**SNV**	0.04	*EGFR*	p. Glu114Ter	c.340G>T
chr7:55221798	**SNV**	0.04	*EGFR*	p. Pro281Leu	c.842C>T
chr7:55221824	**SNV**	0.04	*EGFR*	p. Thr290Ala	c.868A>G
chr7:55221871	**SNV**	0.12	*EGFR*	#N/A	#N/A
chr7:55221872	**SNV**	0.16	*EGFR*	p.?	c.889+27A>G
chr7:55221874	**INDEL**	0.04	*EGFR*	p.?	c.889+32delT
chr7:55221877	**SNV**	0.04	*EGFR*	p.?	c.889+32T>G
chr7:55221881	**SNV**	0.08	*EGFR*	p.?	c.889+36G>T
chr7:55221883	**MNV, INDEL**	0.12	*EGFR*	p.?	c.889+38_889+39insC ...(2)
chr7:55221884	**SNV**	0.04	*EGFR*	p.?	c.889+39T>C
chr7:55221886	**INDEL**	0.04	*EGFR*	p.?	c.889+41_889+42insAG
chr7:55221887	**SNV**	0.04	*EGFR*	p.?	c.889+42T>G

Table (5):Cont'

chr7:55221891	**SNV**	0.04	*EGFR*	p.?	c.889+46C>G
chr7:55221893	**SNV**	0.04	*EGFR*	p.?	c.889+48C>G
chr7:55221894	**SNV**	0.04	*EGFR*	p.?	c.889+49G>T
chr7:55232962	**CNV**	0.04	*EGFR*-AS1, MET, *EGFR*	0	0
chr7:55232962	**CNV**	0.04	*EGFR*-AS1, *EGFR*	0	0
chr7:55233038	**SNV**	0.04	*EGFR*	#N/A	#N/A
chr7:55233052	**SNV**	0.04	*EGFR*	p. Gly601Ala	c.1802G>C
chr7:55241635	**CNV**	0.04	*EGFR-AS1, EGFR*	0	0
chr7:55241637	**SNV**	0.08	*EGFR*	p. Ser695Arg	c.2085T>G
chr7:55241674	**SNV**	0.04	*EGFR*	p. Lys708Glu	c.2122A>G
chr7:55241725	**SNV**	0.16	*EGFR*	p. Thr725Pro	c.2173A>C
chr7:55241728	**SNV**	0.04	*EGFR*	p. Val726Leu	c.2176G>C
chr7:55242412	**SNV**	0.04	*EGFR*	#N/A	#N/A
chr7:55242453	**SNV**	0.08	*EGFR*	p. Pro741=	c.2223C>A
chr7:55248970	**SNV**	0.04	*EGFR*	p.?	c.2284-16C>T ... (2)
chr7:55248973	**INDEL**	0.04	*EGFR*	p.?	c.2284-13_2284-12insATTTATGTGGA ... (2)
chr7:55248978	**SNV**	0.04	*EGFR*	p.?	c.2284-8C>G

Table (5):Cont’					
chr7:55249070	**INDEL**	0.04	*EGFR*	p. Thr790SerfsTer36	c.2369delC
chr7:55249074	**SNV**	0.16	*EGFR*	#N/A	#N/A
chr7:55249078	**SNV**	0.08	*EGFR*	#N/A	#N/A
chr7:55249189	**SNV**	0.12	*EGFR*	#N/A	#N/A
chr7:55249193	**SNV**	0.08	*EGFR*	#N/A	#N/A
chr7:55249194	**SNV**	0.08	*EGFR*	p.?	c.2469+23G>A
chr7:55249198	**SNV**	0.12	*EGFR*	p.?	c.2469+27G>A
chr7:55249210	**SNV**	0.04	*EGFR*	p.?	c.2469+39A>T
chr7:55259541	**SNV**	0.12	*EGFR*	#N/A	#N/A
chr7:55259542	**SNV**	0.12	*EGFR*	#N/A	#N/A
chr7:55259546	**SNV**	0.04	*EGFR*	#N/A	#N/A
chr7:55259548	**SNV**	0.04	*EGFR*	#N/A	#N/A
chr7:55259555	**SNV**	0.04	*EGFR*	p. Ala871=	c.2613A>T
chr7:55259558	**SNV**	0.08	*EGFR*	p. Glu872Asp	c.2616A>T
chr7:55259561	**SNV**	0.04	*EGFR*	p. Gly873=	c.2619A>G
chr7:55259568	**SNV**	0.04	*EGFR*	p.?	c.2625+1G>C
chr7:55259570	**MNV**	0.04	*EGFR*	p.?	c.2625+3_2625+5delinsGCT
chr7:55259574	**SNV**	0.04	*EGFR*	p.?	c.2625+7A>T
chr7:55259577	**MNV, SNV**	0.04	*EGFR*	p.? p.?	c.2625+10_2625+14delinsACCTA, c.2625+12G>T

SNV: Single nucleotide variation, CNV: copy number variation, MNV: multi-nucleotide variant, N/A: not applicable, **INDEL:** insertions/deletion variants.



FFig. (1): Percentage of Predicted Outcome by VEP.

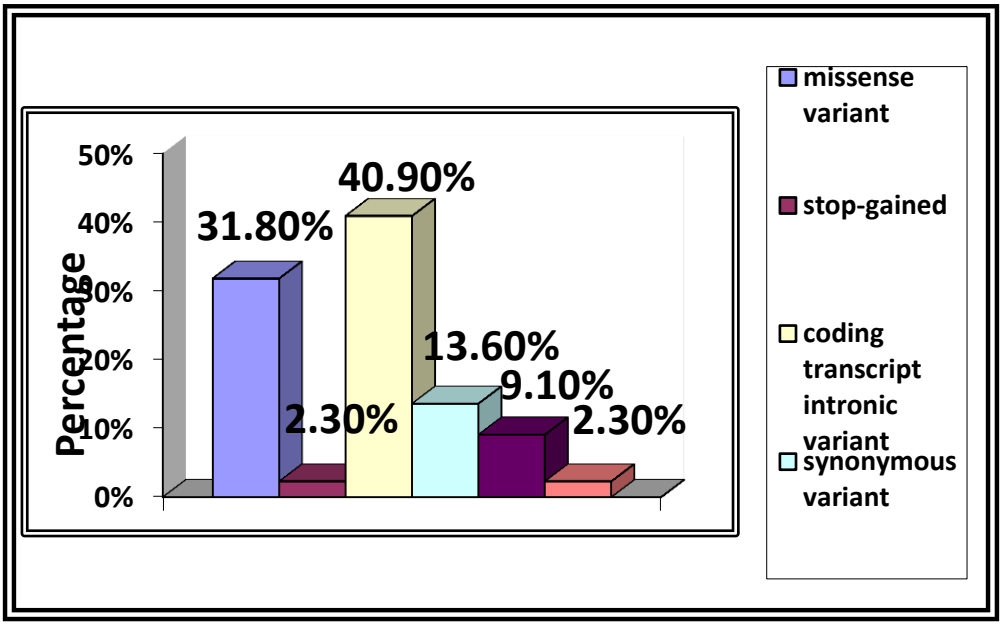


Fig. (2): Variant effect of SNVs

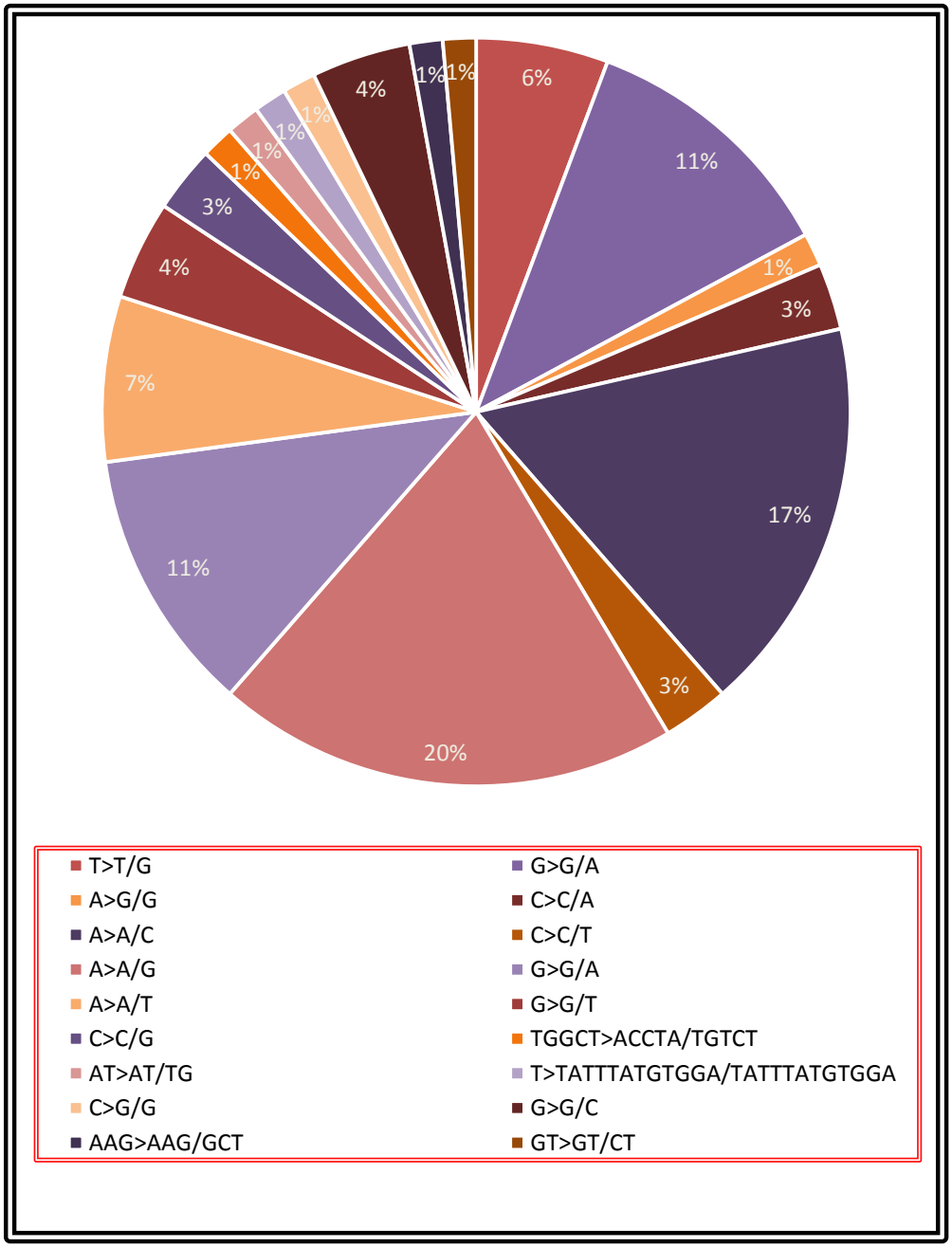


Fig. (3): **Summary of EGFR mutations among HCC patients.**

ASSESSMENT OF MICROBIAL QUALITY AND CHEMICAL CONTAMINATION OF SOME MILK AND MILK PRODUCTS

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Food contamination is the presence of unwelcome pathogens, materials or chemicals that could be harmful to the general public's health. It is an issue that concerns the entire world and has a big impact on every other industry. Depending on the kind of food contamination present, the presence of undesirable substances on food might result in foodborne diseases and other harm. At any point in the food supply chain, food contamination is possible (Hussain, 2016).

Milk's unique composition and properties make it an excellent substrate for bacterial development and a source of bacterial illness. Milk-borne pathogenic bacteria cause over 90% of all dairy-related disorders, posing a severe hazard to human health. The main microbiological risks linked with raw milk consumption are *Staphylococcus aureus*, *Salmonella spp.*, *Listeria monocytogenes*, *Escherichia coli O157:H7*, and *Campylobacter*. Animal health, farming techniques, ambient cleanliness, and insufficient temperature control are all factors that influence the microbiological state of raw milk (Berhe *et al.*, 2020).

Aflatoxins are mycotoxins that are amongst the most toxic mycotoxins and are produced by certain moulds (*Aspergillus flavus* and *Aspergillus parasiticus*) which persist in soil, decaying vegetation, hay, and grains of major concern to the dairy industry. It is known to be one of the most known natural carcinogens. Com-

mercially supplied milk is tested for aflatoxin M1. When M1 aflatoxin levels of 0.5 parts per billion (ppb) or more are detected, milk is rejected because it cannot be used for commodities entering the human food supply. Milk producers sometimes use a level of less than 0.5 parts per billion or 500 parts per trillion as a guideline when choosing to allow milk in the human food supply (Muaz *et al.*, 2022 and Omar, 2016).

There are several types of detection of most of food pathogen such as: Conventional procedures include plating and culture, as well as the use of biochemical assays. Furthermore, immuno-detection has been a popular method for detecting *E. coli O157:H7* since it provides for sensitive and specific detection. In recent years, PCR has grown in popularity as a tool for detecting germs (Kim and Oh, 2020).

Prior studies found Aflatoxin M1 (AFM1) in milk and dairy products using liquid chromatography (LC) with fluorescence detection (FLD) and enzyme-linked immunosorbent assay (ELISA). Nevertheless, there are further techniques, including thin-layer chromatography (TLC), fluorometry, (UHPLCeMS/MS), lateral flow immunoassays, and gel-based immunoassays. High-performance liquid chromatography (HPLC) with fluorescence detection (FD) is commonly used and successfully for the analysis of AFM1. TLC is a very old technique for the separation, purity evaluation, and identification of organic compounds. In fact, it was one of the most widely used separation strategies in previous AF analysis. TLC has been replaced by HPLC with FD, which is now combined with other tools such as MS or GC. The main advantage of using HPLC is the ability to combine different detection systems (fluorescence and UV), allowing identification of many materials from a single sample in addition to the high quality of separation and low Limit of detection (LOD) (Pandey *et al.*, 2021).

Hence, the aim of this study is the assessment of microbial quality and chemical adulteration for some milk and milk products by A) evaluate the microbial contamination in milk and milk products and B) assessment of Aflatoxin M1 in milk and milk products.

MATERIALS AND METHODS

Media and reagents

Different culture media were purchased from Biolife (Italy), Conda (Spain) and Hi-Media (India) and were prepared according to the manufacture recommendation. Water was deionized (DW) in the laboratory using a water purification system from Millipore Milli-Q (USA). All Acetonitrile, acetone, and methanol LC-MS grade were purchased from JT Baker (USA). Aflatoxin M1 reference standards were brought from Dr. Ehrenstorfer (Germany). These solutions cover the range of 0.2–10.0 ng/mL AFM1. Construct the standard curve prior to analysis and check the plot for linearity by examining the correlation coefficient (R2 >0.99) of concentrations and responses.

Methods

Collection of dairy product samples

As shown in Table (1), 25 samples of yoghurt, milk, Karish cheese, old cheese, cheese salad, mesh cheese, Barmili cheese, light salt cheese, chili cheese, Ashura, rice with milk, pudding, and pepper cheese were collected from different markets in Giza governorate and were transferred in an ice box with a monitoring data logger to the microbiological laboratory. A sample of 10 grams was weighed and added to a sampling bag for enumerating contamination for *E. coli*, *Staphylococcus aureus*, Coliform, STEC, and *Salmonella* sp.

Sample preparation

For the pre-enrichment of samples, Buffer Peptone Water (BPW) was prepared by dissolving all components in deionized water, mixed for 10 minutes, and then sterilized. Tryptone Bile Glucuronic Agar (TBX) medium was prepared by dissolving components, adjusting pH according to the manufacture instruction, sterilizing, and cooling. Maximum Recovery Diluent (MRD) was similarly prepared for sample pre-enrichment. Dairy product samples were prepared by adding 10 grams to separate sampling bags followed by the addition of 90 ml BPW and mixing which was equivalent to 10^{-1} dilution.

Enumeration of *E. coli*

After sample preparation, inoculation involved transferring 1 ml of the test sample to Petri plates and test tubes containing MRD. Dilutions were made, followed by adding TBX medium to Petri plates. Inoculated plates were incubated at 44ºC for 24 hours according to the ISO 16649. Enumeration of *E. coli* colonies was done post-incubation, with β-glucuronidase-positive colonies counted.

Enumeration of Coliform bacteria and *Staphylococcus aureus*

Solid selective medium Violet Red Bile Lactose Agar (VRBL agar) and Baird Parker Media (BP) were prepared for coliform bacteria (ISO 4832:2006) and *Staphylococcus. aureus* enumeration (ISO 6888-1:2021) respectively. Inoculation involved transferring samples onto prepared plates, incubating, and counting colonies. Confirmation tests were performed according to what was stated in the ISO methods.

Detection of Enteroinvasive *E. coli* (*ipaH* gene) and Shiga-toxin producing *E. coli* (*stx1*, *stx2* and *eae* genes)

Further isolation, DNA extraction, PCR reaction, and gel electrophoresis were conducted to identify the ipaH gene. Extraction of bacterial DNA was performed using the extraction kit from Biotechon diagnostics (foodproof starprep one kit) ™.

PCR amplification was performed in a 25µL reaction mixture containing 200 ng of DNA template (1µL), 12µL ready to use Mastermix (Deram Taq Green PCR Master Mix, Thermo Fisher scientific), 10 µL of distilled water and 1µL of forward

and reverses primers. DNA amplification was carried out with a thermal cycler (Biorad C-1000, USA) with the following thermal cycling program: initial denaturation at 95ºC for 5min followed by 30 cycles of amplification (denaturation at 95ºC for 30 sec, annealing for 30 sec 61ºC for *ipaH* and extension at 72ºC for 30sec) ending with a final extension at 72ºC for 5 min. Sequence of the forward and reverse primers were designed according to Oscar et al, 2010 (Table 4).

Agarose gel (1%) was prepared by dissolving 1g agarose in 100 ml electrophoresis buffer (TAE, 1X) in microwave. Melted agarose was cooled to 50ºCand ethidium bromide was added at a final concentration of 0.5 mg/ml. The agarose gel was submerged in1X TAE electrophoresis buffer in a horizontal electrophoresis apparatus and DNA samples were loaded, 1 kb DNA ladder (Thermo Scientific) was used as a marker for fragment molecular size determination. Electrophoresis was performed at 80 - 90V, for 30-45 min at room temperature in Biometra power Pack P 25. The gel was visualized by U.V transilluminator (IN Geniuse 3). Additionally, steps for the detection of *Salmonella* spp (ISO 6579-1:2017) and Shiga toxin-producing *E. coli* (STEC) (ISO 13136:2012) were detailed, including pre-enrichment, selective media preparation, inoculation, incubation, and PCR amplification. Detection and interpretation of PCR products were conducted for STEC identification.

Extraction and procedure of AFM1 by Liquid Chromatography (LC)

For each sample, 8.0 g test portion was weighed into a 50 mL centrifuge tube. Then, 22 mL of methanol and 13 mL of water were added. The mixture was shaken at 400 rpm for 10 minutes and then centrifuged at 3000 rpm for 10 minutes. Following centrifugation, the upper oil layer was aspirated and discarded. Subsequently, 30 mL of supernatant was transferred to a 125 mL Erlenmeyer flask and mixed with 60 mL of water. The mixture was passed through glass microfiber paper to collect approximately 60 mL of filtrate (equivalent to about 4.6 g of the test portion) into a 100 mL graduate cylinder for further processing (Manetta, 2011).

IAC isolation

The IAC (Immunoaffinity Chromatography) column, stored at 4ºC, was equilibrated to room temperature for at least 15 minutes prior to use. Following equilibration, the top cap of the column was removed and connected to the reservoir of the column manifold. The bottom cap was also removed, and liquid in the column was allowed to pass through until it was about 2–3 mm above the column bed. Subsequently, 60 mL of the filtrate was passed into the column reservoir, allowing it to flow through the IAC by gravity. After that, 10 mL of water was added to the column reservoir when the liquid level was 2 mm above the column packing. The column was washed with an additional 10 mL of water and allowed to run dry. Then, 10 mL of air was forced

through the column with a syringe. Elution was performed with 0.5 mL of methanol, collecting AFM1 in a 4 mL vial. The column was allowed to run dry, followed by two additional elutions with 0.5 mL of methanol each, collected into the same vial. After allowing the column to run dry again, 10 mL of air was forced through the column. The eluate was evaporated to dryness under a stream of nitrogen at 40°C, followed by the addition of 0.5 mL of LC mobile phase to the residue. After vortexing for 1 minute, 0.05 mL was injected for LC analysis.

1.1.1. LC analysis and AFM1 quantitation and calculation

Prepare standard curves of AFM1 using working standard solutions containing AFM1 covering the range of 0.2–10.0 ng/mL AFM1 (Manetta, 2011). Construct the standard curve prior to analysis and check for linearity by examining the correlation coefficient (R^2 > 0.99) of concentrations and responses. If the test solution area response is outside (higher than) the standard range, dilute the purified test extract with LC mobile phase and reinject it into the LC column. Inject 0.05 mL of reagent blank, AFM1 working standards, or test solution into the LC column. Identify AFM1 peaks in the test solution by comparing the retention time with those of the standards. The retention time of AFM1 was approximately 7 min, and the peaks were baseline-resolved. Quantitate AFM1 by measuring the peak area at the AFM1 retention time and comparing it with the standard curve. Plot the peak area (response, y-axis) of AFM1 standard against the concentration (ng/mL, x-axis) and determine the slope (S) and y-intercept (a). Calculate the level of toxin in the test sample using the formula:

$$\text{Toxin, ng/kg} = ([(R - a)/S] \times V/W) \times F \times 1000$$

Where: R is the test solution peak area, V is the final volume (mL) of the injected test solution, F is the dilution factor (1 when V is 0.5 mL), and W is the weight (4.6 g) of the test sample passed through the IAC edit English if present any wrong.

RESULTS AND DISCUSSION

Microbial contamination in dairy products

Milk and dairy products serve as essential dietary components, providing vital nutrients often challenging to obtain from non-dairy sources. However, these products can also harbor various pathogens, including *E. coli*, Shiga toxin-producing *E. coli* (STEC), coliform bacteria, *Staphylococcus aureus*, and *Salmonella enterica*, which pose significant food safety concerns. In this study, we aimed to evaluate the microbial quality and chemical contamination of selected milk and dairy products obtained from local markets.

Monitoring of *E. coli* bacteria

Escherichia coli, a common indicator of fecal contamination, was detected in 14 out of 25 tested dairy product samples (sample 1, 5, 7, 8, 9, 10, 11, 12, 13, 17,

19, 21, 22, 23 and 24), representing 56% of the total samples as shown in Fig. (1). The highest counts of *E. coli* colonies were observed in Karish cheese (sample 9); while yogurt (sample 5) exhibited the lowest count. Statistical analysis revealed that 44% of the total samples did not contain *E. coli* as shown in Table (2).

Results of this work revealed that *E. coli* is present in tested dairy products (56%) of milk samples, cheese and yogurt, referring to the obtained results in comparison with previous studies, which were higher than El- Barody *et al*. (2022) *E. coli* was detected in 57 samples representing 47.5% of the total examined samples 120 (El-Barody *et al.*, 2022), but they were less than the results of ElMalt *et al*. (2013) *E. coli* was detected in 63 samples representing 63% of the total examined samples 100 (El-Malt *et al.*, 2013), Karish cheese which results the highest amount of *E. coli* agree with El- Barody *et al*. (2022), on the other hand, *E. coli* could not be detected in old cheese samples and Mesh cheese, this was due to the high amount of salts added which acted as natural preservative agent inhibiting pathogenic bacterial growth (Henney *et al*., 2010). The presence of *E. coli* in dairy products was utilized as an indicator of manufacturing environment cleanliness, water quality used in milk product handling and processing, and food handler personal hygiene. In the case of heat-treated dairy products, the pasteurization process can easily kill *E. coli*; thus, the presence of the bacteria in heat-treated dairy products implies that some level of contamination occurred after pasteurization during production and/or packaging (Bagel and Sergentet, 2022). All isolated *E. coli* was identified by PCR, in which all were negative for *ipaH* gene.

Monitoring of Coliform bacteria

Coliform bacteria, another indicator of fecal contamination and overall microbial quality, were identified in 19 out of 25 samples (76%). Among the samples, milk (sample 9) exhibited the highest count of coliform colonies, while Ashura (sample 18) showed the lowest. Confirmation tests showed gas formation in the Durham tube and turbidity in 18 samples, except for sample 12, mesh cheese. Statistical analysis indicated that 72% of the total samples contained coliform bacteria as shown in Table (2).

Coliform count is a traditional indicator of possible fecal contamination, microbial quality, and wholesomeness and reflects the hygienic standards adopted in the food operation. Because coliform organisms are easily killed by heat, these bacteria can also be used as an indicator of heat treatment failure as well as post heat treatment contamination. The presence of coliforms in the analyzed samples indicated a lack of hygienic procedures, incorrect heat processing, or post-pasteurization contamination by handlers (Trmčić *et al.*, 2016). The results showed that 72% of the analyzed samples contained coliform, with 60% of samples having high coliform skipped the Egyptian regulation (> 120 CFU/g).

Monitoring of *S. aureus* and *Salmonella* spp.

After testing *S. aureus* and *Salmonella* in 25 samples of different dairy products, all the samples were not detected as *S. aureus* and *Salmonella* as shown in Fig. (2).

Detection of Shiga Toxin-producing *E. coli* (STEC)

STEC, known for causing severe gastrointestinal illnesses such as hemorrhagic colitis and hemolytic-uremic syndrome, was detected in 12% of the analyzed samples as shown in Fig. (3).

Real-time PCR analysis identified the presence of virulent genes (*stx1*, *stx2*, IAC, and *eaeA*) in three samples: milk (sample 1), Karish cheese (sample 9), and Barmili cheese (sample 13) as represented in Fig. (4). These findings underscore the importance of stringent food safety measures to prevent the transmission of pathogenic bacteria through dairy products

Figure (4). Represent the amplification of *STEC* virulent genes in sample 9 Karish cheese, FAM fluorescence detection of *stx1* gene, VIC fluorescence detection of *stx2* gene, ROX fluorescence detection of *eaeA* gene and cy5 fluorescence are internal amplification control (IAC).

Shiga toxin-producing E. coli (STEC) are thought to be the primary cause of hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (Liao *et al*., 2019). Undercooked meat, unpasteurized dairy products and vegetables, and feces-contaminated water are all plausible routes for STEC human exposure (Dias *et al*., 2022). STEC was identified using a real-time PCR method. The quantitative polymerase chain reaction (qPCR) methodologies are frequently applied in microbiological research to identify the amount and expression of a given target gene, which in this case are the target genes (stx1, stx2, IPC and eaeA). In other words, it is an effective method for measuring gene expression levels. Furthermore, real-time PCR has greater precision, sensitivity, dynamic range, and resolution than classical PCR (Li *et al*., 2017). The current study work agrees with another study reported from Iran by Mohammadi *et al*. (2013), who used PCR to target stx1 and stx2 and then eaeA. This investigation included 206 raw milk samples, 36 of which were determined to be infected with STEC (17%) (Mohammadi *et al*., 2013).

`Detection of Aflatoxin M1

Aflatoxin M1 (AFM1), a mycotoxin commonly found in milk and dairy products. As shown in Table (3), after detection of aflatoxin AFM1using LC, sample 8 represents the highest amount ofAFM1, there are two samples 10 and 16 have low amount of AFM1low of detection by LC and sample 25 not detected as shown in Fig. (5).

The result showed that AFM1 was detected in all samples, with varying concentrations exceeding regulatory limits. While 88% of the samples exceeded Egyptian regulations (0 µg/kg), 52% sur-

passed EU regulations (0.05 µg/kg). These results highlight the need for comprehensive monitoring and control strategies to mitigate the health risks associated with mycotoxin contamination in dairy products.

As milk is used on a big scale by the people, there is an increase in manufacturing of Egyptian raw milk free of mycotoxin. Egypt uses a different standard than other countries such as the USA. The maximum residue limit for AFM1 in raw milk in Egypt is zero, 0.05 µg\L in the EU, and 0.5 µg\L in the US. It was also discovered that an acceptable threshold of risk for AFM1 in fresh raw milk was 0.05 µg/kg , in accordance with Codex Alimentarius and the Joint Expert Committee on Food Additives regulations (JECFA) The current study discovered that all samples under investigation contained aflatoxin M1 at varying concentrations ranging from high to medium to low in all species with 92% of samples detected AFM1, while 8% Exceeding US Limit (0.5 µg\L), and 56% Exceeding EU Limit. According to a different revelation by Anonymous, the European Commission's maximum permitted amount of AFM1 in milk is 0.05 µg\L, (Bakirci, 2001) analyzed 90 raw milk samples for AFM1 and discovered that 87.77% of the positive samples contained aflatoxin M1 and 44.30% of the positive samples above the maximum tolerance limit (0.05 ppb) Approved European Union (EU) (Bakirci, 2001) and ElSayed, *et al*. (2000) who investigated 15 Egyptian cow's milk samples and discovered that three were positive for AFM1 with a mean value of 6.3 ppb (El-Sayed *et al*., 2000). In the current study, we discovered that AFM1 was present in most of samples, although at varying levels ranging from high to medium to low in raw milk and across dairy species. Even at a low level, AFM1 surpasses Egyptian regulations, which state that it should be zero. A similar record explains elevated levels of AFM1 in raw milk because of dairy cows' diets consisting primarily of silage or contaminated feed items. Furthermore, it was discovered that there is a seasonal effect on AFM1 levels, with summer being lower than winter, or that there is a distribution effect due to the long distance between producer and consumer. While low AFM1 concentrations in raw milk in some tests were explained because of mixing and dilution of contaminated milk with less contaminated or non-contaminated milk from various sources. While storage, processing, and fabrication had no effect on AFM1 level (Mahmoudi, 2014).

This study concluded that after testing for the presence of *E. coli*, coliform, *Salmonella* and STEC using selective media, followed by confirmation tests. The tested milk and dairy products collected from local markets in Giza were discovered contaminated with pathogenic bacteria that cause a variety of diseases that impair human health, particularly immunocompromised individuals. Therefore, the high degree of contamination was most likely caused by poor hygiene and the use of unpasteurized milk in dairy product manufacture. Also, Control tech-

niques approved by each country may be able to minimize the limit of aflatoxin in milk and reduce the risk of mycotoxin's influence on human health. So, in Egypt, quality assurance regulations should have been implemented to reduce the quantity of mycotoxins and their negative consequences. The limitations included detecting the presence of more pathogenic bacteria in milk and dairy products, doing PCR with more E. coli genes to identify the pathogenic group that return to, and having limited funding and resources. Furthermore, the experimental time was quite brief, necessitating additional confirmatory studies.

Therefore, it is recommended to conduct further studies to know the types of *E. coli* that are found in milk and cheese and find solutions to get rid of them and do some studies on antibiotics associated with these microbes that are found in milk and cheese.

Conclusion: This study revealed concerning levels of microbial contamination and chemical adulteration in milk and dairy products obtained from local markets. The presence of pathogenic bacteria and mycotoxins underscores the need for stringent food safety protocols, effective regulatory enforcement, and continuous monitoring to safeguard public health. Future research should focus on identifying specific sources of contamination and developing targeted interventions to mitigate risks associated with dairy product consumption. Additionally, efforts should be directed towards enhancing public awareness, improving hygiene practices, and strengthening regulatory frameworks to ensure the safety and quality of dairy products.

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SUMMARY

Food contamination poses a significant threat to public health, potentially causing illnesses due to the presence of infectious organisms like fungi, bacteria, viruses, parasites, or their toxins. Notable bacteria contributing to contamination include *Salmonella* spp., *Escherichia coli*, and *Staphylococcus aureus*, each capable of causing various infections. Additionally, chemical contaminants like Aflatoxin M1 further compound the risks, being known carcinogens. This study aimed to evaluate the microbial quality and chemical contamination of selected milk and milk products. Twenty-five samples were weighed and subjected to enrichment media, followed by culturing on specific selective media for identification of bacteria such as *E. coli*, *Staphylococcus. aureus*, coliforms, and *Salmonella* spp., further confirmed through confirmatory tests. Shiga-toxin producing *E. coli* (STEC) was identified using Real-time PCR, while

PCR and Gel Electrophoresis were utilized to determine the pathogenic group of *E. coli*. Furthermore, liquid chromatography (LC) analysis was conducted to quantify Aflatoxin M1 levels in the samples. Analysis revealed that 56% of tested samples were positive for *E. coli*, while 76% contained suspected coliform colonies, with 72% confirmed. Additionally, 12% of samples harbored STEC, while none contained *Salmonella* or *S. aureus*. Alarmingly, 88% of samples exceeded Egyptian regulations for Aflatoxin M1 (> 0 ug/Kg), with 52% surpassing EU regulations (> 0.05 ug/Kg). These findings underscore significant contamination of milk and dairy products by various bacteria and the presence of excessive Aflatoxin M1 levels, highlighting the urgent need for regulatory measures and quality control in the food industry.

REFERENCES

Bagel A. and Sergentet D. (2022). Shiga Toxin-Producing *Escherichia coli* and Milk Fat Globules. Microorganisms 10. https://doi.org/10.3390/MICROORGANISMS10030496.

Bakirci I., (2001). A study on the occurrence of aflatoxin M1 in milk and milk products produced in Van province of Turkey. Food Control 12, 47-51. https://doi.org/10.1016/S0956-7135(00)00020-7.

Berhe G., Wasihun A. G., Kassaye E. and Gebreselasie K., (2020). Milk-borne bacterial health hazards in milk produced for commercial purpose in Tigray, northern Ethiopia. BMC Public Health 20. https://doi.org/10.1186/S12889-020-09016-6.

Dias D., Costa S., Fonseca C., Baraúna R., Caetano T. and Mendo S., (2022). Pathogenicity of Shiga toxin-producing Escherichia coli (STEC) from wildlife: Should we care? Sci. Total Environ. 812. https://doi.org/10.1016/J.SCITOTENV.2021.152324.

El-Barody, Khater D. F. and Salem A. M., (2022). Bacteriological profiles of chicken meat under different thawing processes. Benha Veterinary Medical Journal Benha Vet. Med. J. 41, 51-55.

El-Malt L. M., Abdel Hameed K. G., and Mohammed A. S., (2013). Microbiological evaluation of yoghurt products in Qena city, Egypt. Vet. World 6, 400-404. https://doi.org/10.5455/VETWORLD.2013.400-404.

El-Sayed A. M. A. A., Neamat-Allah A. A. and Soher E. A. (2000). Situation of mycotoxins in milk, dairy products and human milk in Egypt. Mycotoxin Res. 16, 91-100. https://doi.org/10.1007/BF02946108.

Henney J. E., Taylor C. L. and Boon C. S. (2010). Taste and flavor roles of sodium in foods: A Unique Chal-

lenge to Reducing Sodium Intake. Inst. Med. 67-90.

Hussain M. A. (2016). Food Contamination: Major Challenges of the Future. Foods 5, 1-2. https://doi.org/10.3390/FOODS5020021

ISO 16649-2: (2001). Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli*- Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide.

ISO 6579-1: (2017). Microbiology of the food chain- Horizontal method for the detection, enumeration and serotyping of Salmonella- Part 1: Detection of *Salmonella* spp.

ISO 13136: (2012). Microbiology of food and animal feed- Real-time polymerase chain reaction (PCR)-based method for the detection of foodborne pathogens- Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups.

ISO 4832: (2006). Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of coliforms- Colony-count technique.

ISO 6888-1: (2021). Microbiology of the food chain- Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species)- Part 1: Method using Baird-Parker agar medium.

Kim J. H. and Oh S.W., (2020). Rapid and sensitive detection of *E. coli* O157:H7 and *S. Typhimurium* in iceberg lettuce and cabbage using filtration, DNA concentration, and qPCR without enrichment. Food Chem. 327. https://doi.org/10.1016/J.FOODCHEM.2020.127036.

Li B., Liu H. and Wang W., (2017). Multiplex Real-time PCR assay for detection of *Escherichia coli* O157:H7 and screening for non-O157 Shiga toxin-producing E. coli. BMC Microbiol. 17, 1-13. https://doi.org/10.1186/S12866-017-1123-2/TABLES/4.

Liao Y. Te, Sun X., Quintela I. A., Bridges D. F., Liu F., Zhang Y., Salvador, A. and Wu V. C. H., (2019). Discovery of Shiga Toxin-Producing *Escherichia coli* (STEC)-Specific Bacteriophages from Non-fecal Composts Using Genomic Characterization. Front. Microbiol. 10, 429111. https://doi.org/10.3389/FMICB.2019.00627/BIBTEX.

Mahmoudi R., (2014). Occurrence of Zearalenone in raw animal origin food produced in North-West of

Iran. J. Food Qual. Hazards Control 1: 25-28.

Manetta A. C., (2011). Aflatoxins: Their Measure and Analysis. In: Torres-Pacheco I., editor. Aflatoxins Dtection, Measurement and Control. InTech., Rijeka, Croatia, 93-108. [Google Scholar].

Mohammadi P., Abiri R., Rezaei M. and Salmanzadeh-Ahrabi S., (2013). Isolation of Shiga toxin-producing *Escherichia coli* from raw milk in Kermanshah, Iran. Iran. J. Microbiol. 5: 233.

Muaz K., Riaz M., Oliveira C. A. F. de Akhtar, S., Ali S. W., Nadeem H., Park S. and Balasubramanian B., (2022). Aflatoxin M1 in milk and dairy products: global occurrence and potential decontamination strategies. Toxin Rev. 41, 588-605. https://doi.org/10.1080/15569543.2021.1873387.

Omar S. S., (2016). Aflatoxin M1 Levels in Raw Milk, Pasteurised Milk and Infant Formula. Ital. J. Food Saf. 5, 5788. https://doi.org/ 10.4081/ IJFS.2016.5788.

Oscar G, Gomez D, Octavio A, Delfina U, Jing B., Julio G., *et al.* (2010). Detection of *Escherichia coli* Enteropathogensby Multiplex Polymerase Chain Reaction from Children' DiarrhealStools in Two Caribbean–ColombianCities. Foodborne Pathog Dis., 7:199-206.

Perelle S., Dilasser F., Grout J. and Fach P., (2004). Detection by 50- nuclease PCR of Shiga-toxin producing Escherichia coli O26, O55, O91, O103, O111, O113, O145 and O157:H7, associated with the world's most frequent clinical cases. Mol Cell Probes 18:185-192. doi:10.1016/j.mcp.2003.12.004.

Pandey A. K., Shakya S., Patyal A., Ali S. L., Bhonsle D., Chandrakar C., Kumar A., Khan R. and Hattimare D., (2021). Detection of aflatoxin M1 in bovine milk from different agro-climatic zones of Chhattisgarh, India, using HPLC-FLD and assessment of human health risks. Mycotoxin Res. 37: 265-273. https://doi.org/10.1007/S12550-021-00437-9.

Trmčić A., Chauhan K., Kent D. J., Ralyea R. D., Martin N. H., Boor K. J. and Wiedmann M., (2016). Coliform detection in cheese is associated with specific cheese characteristics, but no association was found with pathogen detection. J. Dairy Sci. 99, 6105-6120. https://doi.org/ 10.3168/JDS.2016-11112.

Table (1): Samples collection from market in Egypt (One sample from each category).

Sample number	Product	Source
1	Milk	Market at Hadayek El Ahram
2	Cheese	Market at Hadayek El Ahram
3	Old cheese	Market at Hadayek El Ahram
4	Salad cheese	Market at Hadayek El Ahram
5	Yoghurt	Market at Hadayek El Ahram
6	Karish cheese	Market at Hadayek El Ahram
7	Milk	Market at Hadayek El Ahram
8	Old cheese	Local market at Giza, (Faisal)
9	Karish cheese	Local market at Giza, (Faisal)
10	Milk	Local market at Giza, (Faisal)
11	Karish cheese	Local market at Giza, (Talbiya)
12	Mesh cheese	Local market at Giza, (Talbiya)
13	Barmili cheese	Local market at Giza, (Talbiya)
14	Light salt cheese	Local market at Giza, (Talbiya)
15	Old cheese	Local market at Giza, (Talbiya)
16	Chili cheese	Local market at Giza, (Dokki)
17	Yogurt	Local market at Giza, (Dokki)
18	Ashura	Local market at Giza, (Dokki)
19	Rice with milk	Local market at Giza, (Dokki)
20	Pudding	Local market at Giza, (Dokki)
21	Barmili cheese	Local market at Giza, (Hadayek el Ahram)
22	Istanbuli Cheese	Local market at Giza, (Hadayek el Ahram)
23	Chili cheese	Local market at Giza, (Hadayek el Ahram)
24	Karish cheese	Local market at Giza, (Hadayek el Ahram)
25	Mesh cheese	Local market at Giza, (Hadayek el Ahram)

Table (2): Enumeration of positive Suspected colonies of *E. coli* and coliform by measuring colony forming unit per gram (CFU/g), in 25 different yogurt and dairy products samples the highest E. coli colonies samples 9 and the lowest colonies sample 5, and the highest coliform colonies sample 9 and the lowest colonies sample 18.

Sample Number	E. coli (CFU/g)	Coliform (CFU/g)	Confirmation
1	88	140	Positive
2	0	0	Negative
3	0	0	Negative
4	0	15	Positive
5	5	20	Positive
6	0	0	Negative
7	100	135	Positive
8	134	28	Positive
9	180	101	Positive
10	15	47	Positive
11	208	304	Positive
12	100	0	Negative
13	205	100	Positive
14	0	45	Positive
15	0	131	Positive
16	8	4	Negative
17	98	98	Positive
18	18	2	Positive
19	0	311	Positive
20	118	0	Negative
21	32	74	Positive
22	99	33	Positive
23	59	105	Positive
24	0	53	Positive
25	0	0	Negative

Table (3): Aflatoxin M1 (AFM1) Levels Detected in Dairy Product Samples Using Liquid Chromatography (LC).

Sample Number	Amount of AFM1 (ppb)
1	0.0563
2	0.1964
3	0.0015
4	0.0289
5	0.2889
6	0.0159
7	0.0222
8	0.6726
9	0.1926
10	< LOQ
11	0.6649
12	0.0138
13	0.0214
14	0.1926
15	0.0230
16	< LOQ
17	0.2132
18	0.1082
19	0.1396
20	0.1142
21	0.0142
22	0.0676
23	0.0939
24	0.4563
25	ND

"< LOQ" stands for "below the limit of quantification" and "ND" stands for "not detected"

Table (4): List of oligonucleotide primers sequences.

target gene name	Forward primer, reverse primer and probe sequences (5 → 3')	Amplicon size (bp)	reference
Stx1	Forward- TTTGTYACTGTSACAGCWGAAGCYTTACG Reverse- CCCCAGTTCARWGTRAGRTCMACRTC Probe- CTGGATGATCTCAGTGGGCGTTCTTATGTAA	131	Perelle *et al*. 2004
Stx2	Forward- TTTGTYACTGTSACAGCWGAAGCYTTACG Reverse- CCCCAGTTCARWGTRAGRTCMACRTC Probe-TCGTCAGGCACTGTCTGAAACTGCTCC	128	Perelle *et al*. 2004
ipah	Forward- CTCGGCACGTTTTAATAGTCTGG Reverse- GTGGAGAGCTGAAGTTTCTCTGC	933	Oscar *et al*. 2010

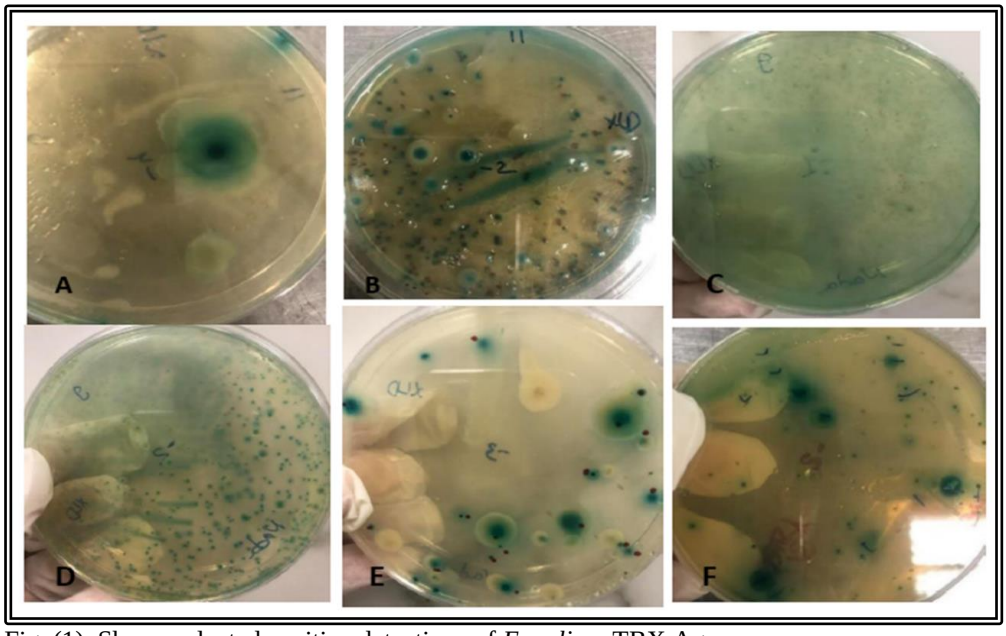

Fig. (1). Shows selected positive detections of *E. coli* on TBX Agar.

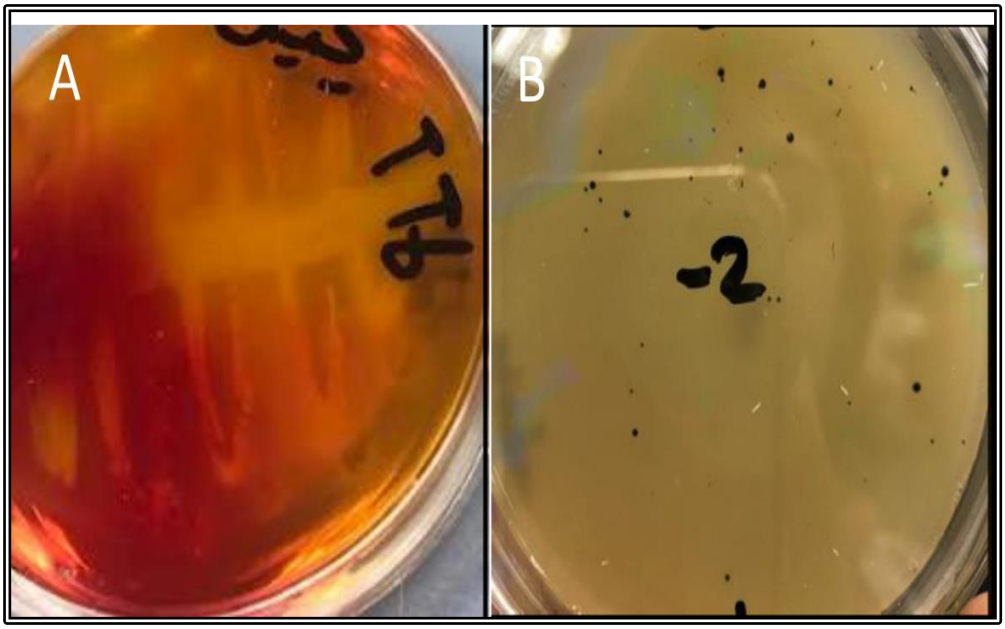

Fig. (2). (A) Shows negative result of *Staphylococcus. aureus* on BP culturing agar and (B) Shows negative detection of suspected colonies of Salmonella spp. on XLD agar.

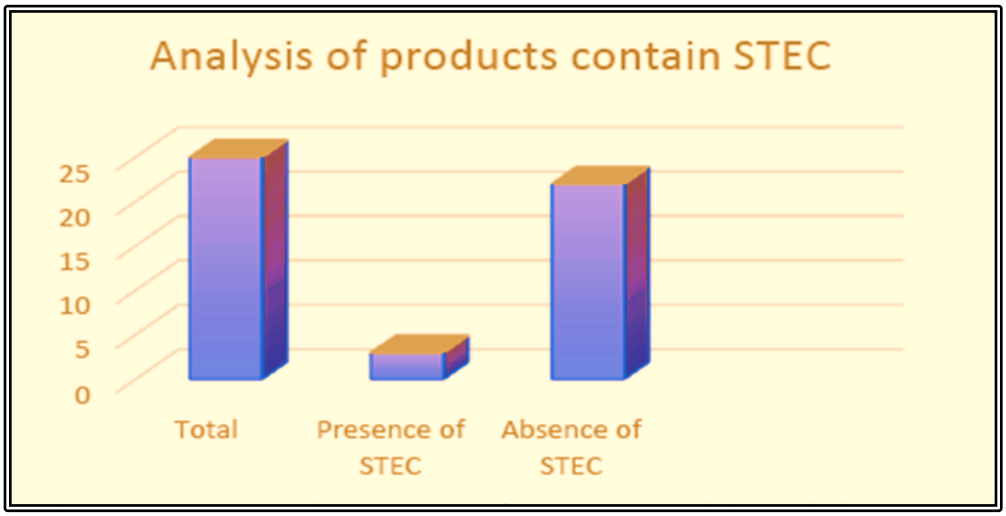

Fig. (3).The presence and absence of STEC in milk and dairy products.

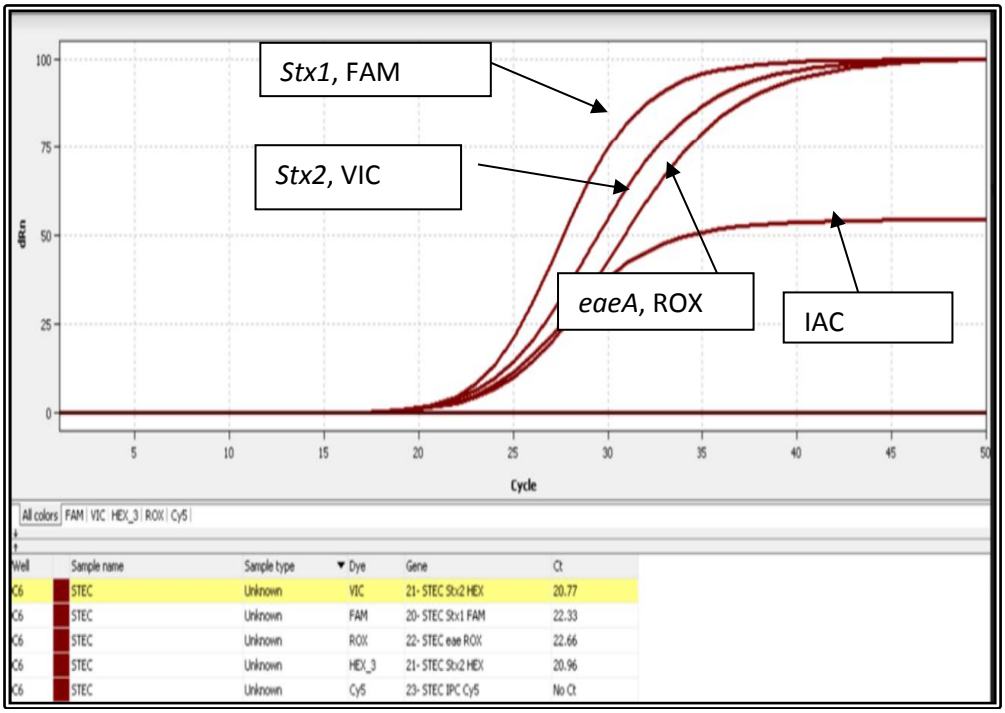

Fig. (4). Represent the amplification of *STEC* virulent genes in sample 9 Karish cheese, FAM fluorescence detection of *stx1* gene, VIC fluorescence detection of *stx2* gene, ROX fluorescence detection of *eaeA* gene and cy5 fluorescence are internal amplification control (IAC).

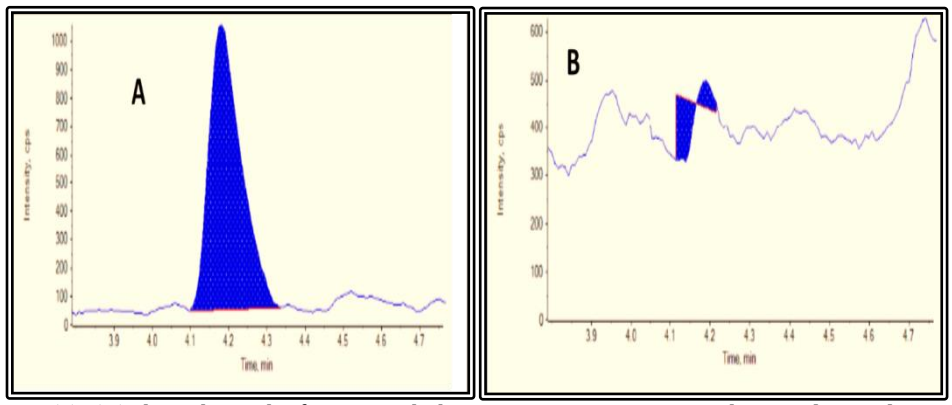

Fig. (5). (A) show the peck of AFM1with the amount 0.6726 ug/Kg in the sample 8 and (B) Show there is no detection of AFM1 in the sample 25.

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