













# Association of Kappa casein gene polymorphism with milk production traits in crossbred dairy cows

## Associação do polimorfismo do gene Kappa-caseína com características de produção de leite em vacas leiteiras mestiças

Wefak J. Albazi<sup>1</sup> , Tahreer M. Al-Thuwaini<sup>3\*</sup> , Muna Alamelty<sup>1</sup> , Zuhair MA. Jeddoa<sup>2</sup> , Rana Mousa<sup>1</sup> , Faten Al-Dawmy<sup>1</sup> , Aqeel H. Atallah<sup>2</sup> , Raced Altaee<sup>1</sup> , Eman Jabber<sup>1</sup> , Fateh Al-Shimmary<sup>1</sup> , Saba Salih<sup>1</sup> , Nabaa Al-Himaery<sup>1</sup> 

<sup>1</sup>College of Veterinary Medicine, University of Karbala, Karbala, Iraq.

<sup>2</sup>College of Health and Medical Techniques, Al-Zahra University, Iraq.

<sup>3</sup>College of Agriculture, Al-Qasim Green University, Babil, Iraq.

\*Corresponding author: [tahrearmohammed@agre.uoqasim.edu.iq](mailto:tahrearmohammed@agre.uoqasim.edu.iq)

### Abstract

Milk's qualitative and technological properties are greatly affected by genetic polymorphisms in the kappa-casein gene, and their polymorphisms may serve as informative markers of yield and composition. Thus, the objective of this study was to detect kappa-casein (*kappa-CN*) gene polymorphisms and their association with milk production traits in crossbred dairy cows. One hundred healthy crossbred (Friesian x Jenoubi) dairy animals between three and five years old were sampled for blood and milk during their first lactation. The genomic DNA was extracted from whole blood, and restriction fragment length polymorphism (RFLP-PCR) was used to determine the genotype of the *kappa-CN* gene. As a consequence of the restriction digestion of this fragment with *Hind* III, it showed three different restriction patterns: BB (453 base pairs uncut), AB (453, 206, and 225 base pairs), and AA (206 and 225 base pairs). Based on genetic diversity, the AB genotype was the most predominant (n = 67), with a frequency of 0.67. A variant genotype of the *kappa-CN* gene was associated with milk production traits in crossbred dairy cows. Animals with the AA variant produced a higher milk yield and a higher percentage of fat, casein, protein, and solids not fat (SNF) ( $P \leq 0.05$ ) (1.397kg, 0.75%, 0.31%, 0.27%, and 0.68%, respectively) than those with the BB variant. A logistic regression analysis confirmed that the *kappa-CN* genotypes increase milk yield and casein content. Therefore, genetic variants of the *kappa-CN* gene could be used as genetic markers for improving milk production traits in dairy cattle.

**Keywords:** cattle, genetic variants, milk protein.

### Resumo

As propriedades qualitativas e tecnológicas do leite são muito afetadas por polimorfismos genéticos no gene kappa-caseína e esses polimorfismos podem servir como marcadores informativos de rendimento e composição. Assim, o objetivo deste estudo foi detectar polimorfismos do gene kappa-caseína (*kappa-CN*) e sua associação com características de produção de leite em vacas leiteiras mestiças. Cem animais mestiços (Friesian x Jenoubi) saudáveis, entre três e cinco anos de idade, foram amostrados durante a primeira lactação para sangue e leite. O DNA genômico foi extraído do sangue total e o polimorfismo dos fragmentos de restrição do DNA genômico (RFLP-PCR) foi usado para determinar o genótipo do gene *kappa-CN*. Em consequência da digestão de restrição deste fragmento com *Hind* III, ele apresentou três padrões de restrição diferentes: BB (453 pares de bases não cortadas), AB (453, 206 e 225 pares de bases) e AA (206 e 225 pares de bases). Com base na diversidade genética, o genótipo AB foi o mais predominante (n = 67), com frequência de 0,67. Genótipo variante do gene *kappa-CN* foi associado com características de produção de leite em vacas leiteiras mestiças. Animais com a variante AA tiveram maior produção de leite e maior percentual de gordura, caseína, proteína e sólidos não gordurosos (SNG) ( $P \leq 0,05$ ) (1,397kg, 0,75%, 0,31%, 0,27% e 0,68%, respectivamente) do que aqueles com variante BB. Uma análise de regressão logística confirmou que os genótipos *kappa-CN* aumentam a produção de leite e o teor de caseína. Portanto, variantes genéticas do gene *kappa-CN* podem ser usadas como marcadores genéticos para melhorar as características de produção de leite em bovinos leiteiros.

**Palavras-chave:** bovinos, variantes genéticas, proteína do leite.

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## 1. Introduction

Bovine milk protein is one of the main parameters for measuring the quality of milk <sup>(1)</sup>. It is primarily composed of four caseins:  $\alpha$ -1,  $\alpha$ -2,  $\beta$ , and kappa-caseins (insoluble fractions) and certain lactoglobulins (soluble fractions) <sup>(2)</sup>. These milk proteins, which include  $\alpha$ s1-casein,  $\alpha$ s2-casein,  $\beta$ -casein, and  $\kappa$ -casein, account for 80% of the total proteins; whey proteins, which consist of  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin, account for 20% of the total proteins. The six proteins listed above make up 95% of the total proteins in bovine milk <sup>(3)</sup>. Milk productivity is associated with milk protein micelles formed by the kappa-casein, which is involved in the formation, stabilization, and aggregation of casein protein micelles and is therefore of great significance in cheese production <sup>(1)</sup>.

Many genes contribute to bovine milk production, and their expression depends on both genetic and environmental factors <sup>(4, 5)</sup>. Among them is the bovine *kappa-CN* gene, which encodes kappa-casein proteins <sup>(7, 8)</sup>. This gene has a 13-kb sequence divided into five exons and is located on chromosome 6 (6q31) <sup>(6)</sup>. Their polymorphisms are important and well-documented due to their influence on the quantitative and technical characteristics of milk and could be used as a predictive molecular marker of milk yield and composition <sup>(2, 8)</sup>.

Several studies on different breeds of dairy cows have shown a relationship between the kappa-casein gene variation and milk yield, fat content, and protein content. Özdemir and Doğru <sup>(9)</sup> studied the effects of *kappa-CN* polymorphism on milk production traits in Brown Swiss, Holstein, and East Anatolian Red cows. This study provided evidence that fat yield and percentage can be increased by increasing the frequency of *kappa-CN* BB genotypes, whereas milk production can be maximized by increasing *kappa-CN* AB genotypes with selective breeding in herds. A higher frequency of *kappa-CN* BB genotypes in herds can also increase yield traits significantly. Furthermore, it was demonstrated that Holstein and Girolando cows with AB and BB genotypes of the *kappa-CN* gene had a higher milk fat content when compared to cows with AA genotypes <sup>(10)</sup>. Two allelic variants, A and B, of the bovine *kappa-CN* (*CSN3*) gene are determined by missense mutations in exon IV. The amino acids 136 and 148 differ between variants A and B. For A and B, threonine is replaced by isoleucine at position 136, and aspartic acid by alanine at position 148, respectively <sup>(11)</sup>. In the study by Mir et al. <sup>(12)</sup>, the effect of s1 casein,  $\beta$ -casein, kappa-casein,  $\alpha$ -lactalbumin,  $\alpha$ -lactoglobulin, and  $\beta$ -lactoglobulin variations on milk yield was measured. Higher milk production was associated with the AB genotype identified in the *kappa-CN* gene. A polymorphism g.10888T>C could lead to the

substitution of isoleucine for threonine in the kappa-CN protein in Chinese Holstein cattle that is associated with milk production traits <sup>(13)</sup>. In another study, PCR-RFLP analysis was used to test for genetic variants of the  $\alpha$ S1-casein gene in Bulgarian Rhodopean cattle and their association with milk production and quality. The data showed that the three genotypes BB, CC, and BC accounted for 26.4%, 2.3%, and 71.3% of the population, respectively. Genetic variant BC was linked to cow milk production and milk butter, and genotype CC to fat and protein content <sup>(14)</sup>. Based on the genetic variation on milk protein, Kyselová et al. <sup>(15)</sup> investigated the influence of  $\beta$ - and  $\kappa$ -casein and  $\beta$ -lactoglobulin genotypes in Czech Fleckvieh cattle on important milk physiochemical and technological characteristics such as acidity, alcohol stability, the contents of some minerals, and the parameters of acid fermentation ability (FEA). A significant association between genotype *CSN3* and alcohol stability (AS) was found ( $P < 0.05$ ). As reported by Čítek et al. <sup>(16)</sup>, *CSN3*, *CSN2*, and *LGB* polymorphisms were associated with milk yields and fat and protein production for selected cattle breeds in the Czech Republic. Although the polymorphisms of the *kappa-CN* gene have been studied, little information is available on how they affect milk production in crossbred dairy cows. This study investigated the frequency of *kappa-CN* polymorphisms in crossbred dairy cows and their association with milk production traits.

## 2. Material and methods

### 2.1 Animals, phenotypic data, and milk collection

The study was conducted at Karbala University during the period from September 2019 to February 2020, following the international recommendations for the care and use of dairy cattle <sup>(17)</sup>, with approval number (Vet, No. 020,9,19). Crossbred (Friesian x Jenoubi) dairy cows with known pedigrees were included in the study. The study included 100 healthy crossbred dairy cows, aged between three and five years old; feeding and maintenance were identical for all animals. The data were classified according to parity (lactation I, II, III, and IV). All cows completed their lactations (305 days). Phenotypic data were collected from the laboratory of the dairy herd in Wasit Province, which included their age, milking records, lactation days, calving records, and sire and dam identification. In the studied herd, 2 sires were randomly allocated to mate with about 25–30 dams. A sample of 10 milliliters of milk was taken from each dairy cow and evaluated by an industrial scale milk analyzer (EKOMILK) to examine the fat, protein, casein, and solid portions of each milking. Descriptive statistics for the milk production trait are presented in Table 1.

**Table 1.** Descriptive statistics for milk productive traits in crossbred dairy cows.

Trait	n	Mean	SD	Minimum	Maximum
Milk yield in kg	100	10.185	311.04	8.64	12.167
Fat%	100	4.69	0.63	2.70	7.14
Casein%	100	2.71	0.16	1.94	4.12
Protein%	100	3.05	0.24	2.16	4.62
SNF%	100	7.65	0.32	5.04	9.31

SD = standard deviation, SNF = solids not fat.

### 2.1 DNA extraction and PCR-RFLP

Blood samples were collected from crossbred dairy cow flocks at the cattle station northeast of rural areas in Iraq (Waist Province). Blood was drawn and placed into tubes containing EDTA for genetic analysis. DNA was extracted from whole blood with a gSYNCTM DNA extraction kit (Geneaid, New Taipei City, Taiwan), following the manufacturer’s instructions, and then measured on a Nanodrop spectrophotometer (Biodrop, UK) at 260/280 absorbance ratios (18). Polymerase chain reaction (PCR) was used to amplify a 453 bp fragment containing most of the exon IV coding region of the bovine *kappa-CN* gene. The primers for amplification of the *kappa-CN* gene fragments were reported by Barroso et al. (19), and their nucleotide sequences were as follows: (F) TGTGCTGAGTAGGTATCCTAGTTATGG and reverse (R) GCGTTGTCTTCTTTGATGTCT CCT. The PCR reaction was conducted using the Bioneer PCR premix (50 µM dNTPs, 10mM Tris-HCl (pH 9.0), 30mM KCl, 1.5mM MgCl2, and 1 U Top DNA polymerase) (20). In the PCR setup, the following conditions were used: denaturation at 94 °C for 4 min, 35 cycles of annealing, elongation, and extension (94 °C, 65 °C, and 72 °C for 1 min), and a final extension (72 °C for 5 min). Results were verified by electrophoresis of PCR products on 1.5% agarose gels and determination of gel images with a Chemidoc Gel Imager (Bio-Rad, USA). The PCR product was digested with *Hind* III restriction enzyme for the genotyping *kappa-CN* gene. Gene fragments were digested with restriction enzymes in a total volume of 20 µl (10 µl PCR product, 1 X enzyme buffers, 5 U enzymes, and distilled water) and subsequently incubated at 37 °C for 5 h. Electrophoresis on 2% agarose gels was used to detect restriction products (21).

### 2.3 Statistical analysis

Genotype and allele frequency were calculated using PopGen32, version 1.31 (22). Hardy-Weinberg equilibrium was calculated using the HWE law. Polymorphism information content (PIC) was calculated according to Botstein et al. (23). Using the SPSS statistical program (SPSS 23.0 (NY, USA)), the following general linear model was used:

$$Y_{ijklm} = \mu + G_i + P_j + A_k + LM_l + DIM_m + e_{ijklm}$$

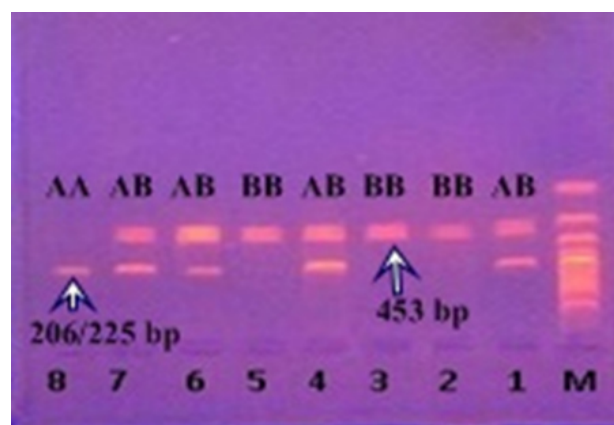
where  $Y_{ijklm}$  = the analyzed trait of each cow,  $\mu$  = the mean of milk production traits,  $G_i$  = fixed effect of  $i^{th}$  genotypes ( $i = AA, AB, BB$ ),  $P_j$  = fixed effect of  $j^{th}$  parity ( $j = 1, 2, 3, 4$ ),  $A_k$  = fixed effect of  $k^{th}$  age group (3-4, >4-5),  $LM_l$  is the fixed effect of the  $l^{th}$  lactation month ( $l = Dec., Nov.,$  and  $Oct.$ ),  $DIM_m$  = days in milk as a covariate, and  $e_{ijklm}$  = random residual error. The Bonferroni adjusted p-values were used to evaluate statistically significant differences between means ( $P \leq 0.05$ ). Logistic regression analysis with the significance level set at  $P \leq 0.05$  was used to confirm the association between *kappa-CN* polymorphisms and milk production traits according to the following model:  $Logit(\pi) = \alpha + G_i + P_j + A_k + LM_l + \beta_{DIM}$ ;

where  $\pi$  is the probability of milk production traits,  $\alpha$  is the scale parameter of the trait,  $G_i$  is the fixed effect of the  $i^{th}$  genotype ( $i = AA, AB, BB$ ),  $P_j$  is the fixed effect of the  $j^{th}$  parity ( $j = 1, 2, 3, 4$ ),  $A_k$  = fixed effect of  $k^{th}$  age group (3-4, >4-5),  $LM_l$  is the fixed effect of the  $l^{th}$  lactation month ( $l = Dec., Nov.,$  and  $Oct.$ ), and  $\beta_{DIM}$  is the covariate factor of the days in milk.

## 3. Results

### 3.1 Genotyping of *kappa-CN* gene and genetic diversity

PCR-RFLP analysis was performed on all samples using the *Hind* III restriction enzyme to assess variations in the *kappa-CN* gene. *Hind* III distinguished the two alleles, A and B, the latter remaining undigested (Figure 1). Through PCR-RFLP analysis, the A and B alleles of *kappa-CN* were identified by amplification of a 453 bp fragment located across exon IV. Following restriction digestion, three restriction patterns were identified, referred to as BB (uncut 453 bp), AB (453 bp, 206, and 225 bp), and AA (206 and 225 bp) (Figure 1).



**Figure 1.** Restricted product analysis of *kappa-CN* gene from crossbred dairy cows showing three genotype AA, AB, and BB on 2% agarose gel with *Hind* III endonuclease enzymes. M: 100 bp ladder, 1, 4, 6, and 7 represented AB, 2, 3, and 5 represented (BB), 8 represented (AA).

A study of the genetic diversity of *Hind* III -RFLP polymorphisms of the *kappa-CN* gene found that AB was the predominant genotype ( $n = 67$ ), with a total frequency of 0.67. A total frequency of 0.20 ( $n = 20$ ) was also determined for the AA genotype, and a frequency of 0.13 ( $n = 13$ ) was determined for the BB genotype (Table 2). Considering information about polymorphism content

(low polymorphism if PIC value  $< 0.25$ , moderate polymorphism if  $0.25 < \text{PIC value} < 0.5$ , and high polymorphism if PIC value  $> 0.5$ ), this study provided a moderate amount of information about the polymorphism. Based on the Chi-square test, the *kappa-CN* polymorphism appeared to significantly deviate from HWE ( $P \leq 0.05$ ) (Table 2).

**Table 2.** Genetic diversity of the *kappa-CN* gene in crossbred dairy cows

Observed genotypes			Genotype frequencies			Allele frequencies		<i>H<sub>o</sub></i>	<i>H<sub>e</sub></i>	<i>N<sub>e</sub></i>	PIC	$\chi^2$
AA	AB	BB	AA	AB	BB	A	B					
$n=20$	$n=67$	$n=13$	0.20	0.67	0.13	0.53	0.46	0.67	0.50	1.99	0.35	11.66

Abbreviations: *n* – number of individuals, *H<sub>o</sub>* – observed heterozygosity, *H<sub>e</sub>* – expected heterozygosity, *N<sub>e</sub>* – effective allele frequency, PIC – polymorphism information content,  $\chi^2$  – Chi-square. All Chi-square tests have two degrees of freedom and are within the significance level  $P \leq 0.05$ .

### 3.2 Association analyses of the *kappa-CN* gene

There were significant ( $P \leq 0.05$ ) differences between the AA, AB, and BB genotypes concerning milk production traits. Animals with AA genotypes showed higher milk yield, percentage of fat, and solids not fat (SNF) ( $P \leq 0.05$ ) than animals with AB and BB genotypes

(Table 3). Univariate regression analyses further explored the association of *kappa-CN* genotypes with milk production traits (Table 4). The higher associated factors in milk yield and casein content were identified by multiple regression analyses, which indicated that the *kappa-CN* genotype had a beneficial influence on milk yield and casein content.

**Table 3.** Association of *kappa-CN* genotype and milk production traits in crossbred dairy cows.

Genotypes	LSM $\pm$ SE				
	Milk yield (kg)	Fat%	Casein%	Protein%	SNF%
AA	10986 $\pm$ 226 <sup>a</sup>	5.03 $\pm$ 0.19 <sup>a</sup>	2.60 $\pm$ 0.06 <sup>a</sup>	3.11 $\pm$ 0.09 <sup>a</sup>	8.42 $\pm$ 0.11 <sup>a</sup>
AB	9754 $\pm$ 241 <sup>ab</sup>	4.64 $\pm$ 0.24 <sup>ab</sup>	2.53 $\pm$ 0.12 <sup>a</sup>	3.08 $\pm$ 0.10 <sup>a</sup>	8.09 $\pm$ 0.29 <sup>ab</sup>
BB	9589 $\pm$ 123 <sup>b</sup>	4.28 $\pm$ 0.18 <sup>b</sup>	2.29 $\pm$ 0.10 <sup>b</sup>	2.84 $\pm$ 0.13 <sup>b</sup>	7.74 $\pm$ 0.31 <sup>b</sup>
<b><i>P</i>-value</b>	<b>0.01*</b>	<b>0.02*</b>	<b>0.03*</b>	<b>0.04*</b>	<b>0.03*</b>

Least square means  $\pm$  standard error. \* ( $P \leq 0.05$ ). <sup>ab</sup> Significant differences in means represent differences in the same column within each classification, SNF = solids not fat.

**Table 4.** Logistic regression analysis of *kappa-CN* genotype and milk production traits in crossbred dairy cows.

Characteristic	Univariate logistic regression			Multivariate logistic regression		
	Estimate	Odds ratio (95% CI)	<i>P</i> -value	Estimate	Odds ratio (95% CI)	<i>P</i> -value
Milk yield (kg)	0.94	2.55 (1.14-4.06)	0.02	1.03	2.80 (0.81-4.93)	0.03
Fat%	0.67	1.95 (0.51-3.45)	0.03			
Casein%	1.04	2.82 (1.43-3.72)	0.01	1.11	3.03 (0.78-4.91)	0.01
Protein%	0.42	1.52 (0.63-2.86)	0.03			
SNF%	0.37	1.44 (0.71-3.46)	0.02			

The *P*-value with statistical significance is indicated in bold numbers; CI: confidence interval.

## 4. Discussion

A Chi-square test showed that the distribution of *kappa-CN* genotypes deviated from the Hardy-Weinberg equilibrium ( $P \leq 0.05$ ). In livestock, genetic variation in the *kappa-CN* gene has been reported in several studies. Mir et al. (12) investigated the effect of variation in  $\alpha$ -s1 casein,  $\beta$ -casein, kappa-casein,  $\alpha$ -lactalbumin, and  $\beta$ -lactoglobulin genes on milk yield.

Milk protein genes are polymorphic, with  $\alpha$ -casein C exhibiting a high allele frequency of 0.51,  $\beta$ -casein A showing 0.93,  $\kappa$ -casein A displaying 0.92, and  $\alpha$ -lactalbumin B exhibiting 0.93; while  $\beta$ -lactoglobulin B exhibited a 0.91 allele frequency. Maletić et al. (24) used specific primers to identify genotypes of  $\kappa$ -casein. The digestion of fragments with specific endonucleases revealed three genotypes in Holstein Friesian cows: AA, AB, and BB, while only two genotypes were detected in



Busha cows: AA and AB. In the AA genotype, the fragments were 156, 132, and 91 base pairs; in the AB genotype, the fragments were 288, 156, 132, and 91 base pairs; while in the BB genotype, the fragments were 288 and 91 base pairs. Concerning Holstein and Girolando cows, Kyselová et al. <sup>(15)</sup> investigated the level of polymorphism in the  $\beta$ - and  $\kappa$ -casein and  $\beta$ -lactoglobulin genes. In Holstein cows, the frequencies of genotypes AA, AB, and BB of  $\kappa$ -casein are 66.83, 31.84, and 1.33 %, respectively, whereas in Girolando cows, the respective frequencies are 71.38, 27.90, and 0.72 %. Holstein cows (0.827 and 0.173) and Girolando cattle (0.853 and 0.17) had higher frequencies of the A allele than the B allele. Our results agree with the findings of <sup>(25, 26)</sup>, who reported the predominance of the AB genotypes at 0.30 in Iranian Holstein cattle and 0.48 in Mexican Jersey cattle.

Association analysis of this study showed that individuals with the AA genotype had higher protein and casein, fat content, milk yield, and SNF than those with the AB genotype and BB genotype. The reason for this is that dairy cows possess two gene families that determine the properties of milk production, namely casein and whey <sup>(5)</sup>. In bovine milk, casein is the main protein. It exists in several molecular forms. These proteins affect milk production and milk components <sup>(8)</sup>. Casein genetic polymorphisms are crucial for milk production traits and have received considerable attention <sup>(2)</sup>. Several studies have found that the AA genotype is also linked to higher milk production. Curi et al. <sup>(27)</sup> reported that the  $\kappa$ -casein genotype AA is associated with higher milk production than the genotype BB, with heterozygous AB being intermediate. In addition, Trakovická et al. <sup>(28)</sup> indicate that genotype AA is positively associated with milk, protein, and fat yields, and the A allele is positively associated with milk production traits. In this regard, Zepeda-Batista et al. <sup>(29)</sup> found that genotype AA in the *CSN3* gene resulted in increased milk production in Holstein and Jersey breeds. The favorable effect of the AA genotype in the kappa-casein gene on proteins might be explained by amino acid differences in the mature protein, which may affect the biological properties of kappa-casein and its interactions with the other fractions in the casein micelles <sup>(11)</sup>. A genetic variation in kappa-casein may also indicate that the mature protein is associated with other polymorphisms in non-coding sequences (promoter). A mutation could cause some alleles to express differently, such as the two main alleles of bovine cattle's kappa-casein gene <sup>(30)</sup>.

## 5. Conclusions

Kappa-casein affects milk yield, protein levels, and fat contents. The *CSN3* A variant is associated with milk production characteristics in crossbred dairy cows. Animals with AA genotypes produced higher milk yields

with higher protein and fat percentages than those with AB and BB genotypes. This study demonstrated that the  $\kappa$ -casein gene could be used as a genetic marker in gene-assisted selection programs to improve milk production traits in dairy cattle.

### Conflict of interest

The authors declare that there are no conflicts of interest.

### Author contributions

*Conceptualization*: W. J. Albazi. *Data curation*: F. Al-Dawmy and Z. MA. Jeddoa. *Investigation*: R. Mousa, M. Alamelly, A. H. Atallah, R. Altaee, E. Jabber, F. Al-Shimmary, S. Salih and N. Al-Himaery. *Methodology*: R. Mousa, M. Alamelly and A. H. Atallah. *Supervision*: W. J. Albazi. *Writing (original draft)*: W. J. Albazi and T. M. Al-Thuwaini. *Writing (review and editing)*: T. M. Al-Thuwaini.

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