Bulletin of the National Research Centre

RESEARCH

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Sequence analysis of *TLR4* gene in river buffalo (Egyptian breed) and SNPs association with Mastitis

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Abstract

Background Mastitis is a major cause of economic loss for dairy farmers and industry. River buffalo plays an economically significant role in Egypt. Buffaloes with mastitis have reduced milk yield and change in milk composition. Genetic variations in the Toll-like receptor4 (TLR4) gene have been related to several diseases in farm animals and humans including mastitis. The present investigation aims to find the genotypic variations in the TLR4 gene and their relation to mastitis in the river buffalo, Egyptian breed.

Results DNA from 30 buffaloes' blood samples (15 healthy and 15 with mastitis) were extracted and the TLR4 gene was sequenced. Twenty-one Single nucleotide polymorphisms (SNPs) were found from which four SNPs were associated with mastitis: one in 5'UTR (c.1-g27) and 3 SNPs in the coding region at c.87, c.575, and c.576. The nucleotide variations in SNPs c.1-g27(C > A) and c.87 (C > A) were only present in buffalo with mastitis, while buffaloes with genotype CC at both locations were healthy. The AA genotype at c.87 (P.29) results in a stop codon leading to an abnormally shortened protein. The nonsynonymous SNPs c.575 A > G and c.576 T > G shared amino acid 192 resulting in three amino acids (His192Arg/Gln). The dominant genotypes AA at c.575 and TT at c.576 were associated with mastitis resistance (OR < 1.00), while recessive genotype GG at c.575 was associated with mastitis susceptibility (OR > 1.00). These two SNPs may affect their role in ligand recognition since they were in the LRR4 domain (p.174–p.197) which is part of coreceptor binding region 1.

Conclusions The present study confirms the relation between TLR4 genotypes and mastitis resistance or mastitis susceptibility in Egyptian dairy buffalo. The study suggested four SNPs (c1-g27C > A, c.87 C > A, c.575A > G, and c.576 T > G) have the potential to be markers for assisted buffalo selection to improve milk production.

Keywords TLR4, Egyptian buffalo, Mastitis, SNPs

Background

River buffalo plays an economically significant role in Egypt with a population of 3.7 million heads (FAO 2017). They supply about 45% and 37% of total milk and red meat produced, respectively (FAO 2019). In Egypt, small-scale farmers own 70% of the total livestock population (Aidaros 2005). Buffalo's genetic improvement, especially in disease resistance, reproductive performance, and quantity of meat and milk production is of foremost importance among agricultural research needs.

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Mastitis in farm animals is a major cause of economic loss for dairy farmers and industry, the prevalence of mastitis in the buffalo Egyptian breed amount to 43.5% (Elhaig and Selim 2015) whereas in the Brazilian Murrah breed it was 42.8% (Cerón-Muñoz et al. 2002). The inflammation caused by mastitis reduces milk yield and changes the milk composition, e.g., reduction in fat, casein proteins, and calcium content with a simultaneous increase in the concentration of whey proteins, sodium, and chlorine (Jóźwik et al. 2012).

Severe acute inflammation or chronic mastitis results from suboptimal and dysfunctional mammary defenses. Clinical mastitis is recognized by abnormal milk, gland swelling, and /or systemic illness, whereas subclinical mastitis is characterized by apparent normal milk with an increase in somatic cell count (SCC) and reduction in milk production which may account for 70–80% of the total losses (Philpot and Nickerson 1991; Philip et al. 1993; Seegers et al. 2003). Mastitis is caused by two major pathogens Escherichia coli and Staphylococcus aureus (Kempf et al. 2016; Silva et al. 2016; Saglam et al. 2017.) with *E. coli* elicits a strong and earlier response, through the innate immune gene.

The innate immune system is the first line of defense against invading pathogens (Tarazona-Santos et al. 2013). It gives the mammary gland the ability to combat a variety of invading pathogens due to the rapid response even upon first exposure (Oviedo-Boyso et al. 2007). Innate immunity genes are associated with the severity of mastitis and the clinical manifestation commonly seen in dairy cows infected with this pathogen.

Toll-like receptor4 (TLR4), a transmembrane protein member, a member of the toll-like receptor family plays an important role in mastitis. It recognizes lipopolysaccharide (LPS) present in several gram-negative bacteria and some gram-positive bacteria (Moroni et al. 2014; Ciesielska et al. 2021). Activation of TLR4 leads to an intracellular signaling pathway NF-kB and inflammatory cytokine production responsible for activation of the innate immune system. TLR4 is one of the best-studied TLR members, its structure consists of three domains: an extracellular leucine-rich repeat (LRR) domain, a transmembrane domain, and an intracellular Toll-interleukin-1 receptor (TIR) domain (Uematsu and Akira 2008; Lu et al. 2008).

TLR4, a candidate gene responsible for the first recognition of invading organisms, is highly polymorphic. In cattle TLR4 nucleotide variations and somatic cell score (SCS) in milk were associated with mastitis (Sharma et al. 2006; Li et al. 2014; Wang et al. 2007; Gupta et al. 2015). Deb et al. (2013) reported thirty-six single nucleotide polymorphisms (SNP) in cattle across fourteen breeds.

In buffalo, Culhane and Sanguan (2012) associated the genetic variations in TLR4 and mastitis in the Murrah breed using the RFLP technique. They reported two genotypes AA and AB of single mutation C > G that causes a substitution of Arginine by Guanine. TLR4 polymorphism was also associated with other buffalo diseases such as endometritis (Osman et al. 2018) and tuberculosis (Alfano et al. 2014) and with severe sepsis or septic shock in humans (Nachtigall et al. 2014).

The present study aimed to investigate the association of the genetic variations in the TLR4 gene in river buffalo (Egyptian breed) and mastitis. Genetic identification of buffaloes with high resistance to mastitis and other diseases will help in selecting breeds with high resistance to microbial diseases to improve productivity. Buffalo's genetic improvement is of foremost importance among agricultural research needs.

Methods

Blood samples from thirty buffaloes (15 healthy and 15 with mastitis) were collected on EDTA from Governmental farm in Kafr El-Sheikh, Egypt, by the farm veterinarian. Genomic DNAs were extracted using the salting-out method according to Miller et al. (1988). The final DNA concentrations for polymerase chain reaction (PCR) were 50 ng/µl.

Polymerase chain reaction (PCR)

Five primer pairs were designed to amplify the coding region of the TLR4 gene based on the Bubalus bubalis sequence (acc#JN786600.1) using the Primer 3 Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). Table 1 lists the primers and their annealing temperatures and the target lengths. TLR4 has three exons. Primer 1 covers 5′UTR, exon 1, and partial 5′ intron1; primer 2 covers partial 3′ intron1,

Table 1 Five Primers used to amplify the TLR4 gene

Primer	5′-sequence-3′	Ann. temp	Target length
1	F1: GACAGCCATCTATAAGCCAAGG R1: TGTCTGTTTGCAAATGAACCT	53 °C	785 bp
2	F 2: AGAGTTGCTGGGAAGTCTGC R2: AACATTCCTCCTTGTACA GTGGT	56 °C	273 bp
3	F3: GCATTGTTATATCTGTGTGGA GACC R3: GAGATCTAGATACTGAAGGCT TGG	55 °C	985 bp
4	F4: CGAATTCTCAGGGGACGATA R4: GCTCTGCACACATCATTTGC	54 °C	953 bp
5	F5: GACTGCAGTTTCAACCGTATCA R5: TGGCAGCATTTACTTGTTAAC TGA	55 °C	953 bp

exon 2, and partial 5′ intron2; primers 3, 4, and 5 covers partial 3'intron2 exon 3 and 3'UTR.

Coding regions of the TLR4 gene were amplified by polymerase chain reaction (PCR) assays, in a final volume of 50 µl consisting of 25.5 µl of nuclease-free water, 5 µl of 10X DreamTaq™ DNA polymerase buffer, 5 µl (100 µM) dNTPs, 5 µl of each primer (20 µM), 0.5 µl (5U/µl) of DreamTaq™ DNA polymerase (Fermentas, Waltham, USA) and 4 µl genomic DNA (50 ng/µl).

The PCR reactions run for thirty-five cycles in a Q-Cycler, (HVD life sciences, Wien, Austria) preceded by first denaturation at 95 °C for 5 min; 1 min denaturation at 95 °C; 2 min annealing at 53–56 °C, 2 min extension at 72 °C, ended by a final extension at 72 °C for 10 min. The target PCR products were purified using a MEGA quick-spin TM total fragment DNA purification Kit (iNtRON Biotechnology, Gyeonggi-do, South Korea) and sequenced two ways (Macrogen, Seoul, South Korea).

Sequence analysis and genetic association

The Polymorphic sites were determined by visual examination. Multiple alignments analysis CLUSTAL–Omega program was used (https://www.ebi.ac.uk/Tools/msa/clustalo/).

To associate buffalo genotypes and mastitis, the detected SNPs were tested for deviation from Hardy–Weinberg equilibrium (HWE) using the Fisher Exact test. A univariate logistic regression model performed for SNPs that were significantly associated with the disease. Statistical analysis was performed using the R program, and P-value was corrected using the Bonferroni method (Bland and Altman 1995). The Odds Ratio test (OR) was calculated with a 95% confidence interval.

Protein prediction and analysis

The protein sequence was predicted using EMBOSS Six-pack (https://www.ebi.ac.uk/Tools/st/emboss_sixpack/) and the possible SNPs-based amino acids substitutions were evaluated. SignalP-5.0 software (http://www.cbs.dtu.dk/services/SignalP/) is used to predict the cleavage sites and signal peptides. SMART analysis (http://smart.embl-heidelberg.de/) was used to detect the protein domains of the TLR4 gene.

Results

Buffaloes' TLR4 genomic sequences were assembled using five overlapping amplified PCR products resulting in 3426 bp and were submitted to GenBank (acc. # MN394471.1). They cover the full coding region (2526 bp), partial regions of 5′UTR (592nt), exon1 (93nt), 5′intron1 (99nt), 3′intron1 (70nt), exon2 (167nt) 5′intron2 (25nt), 3′intron2 (88nt), exon3 (2266) and 3′UTR (26). The open reading frames encode 841 amino acid residues covering the 3 exons.

Sequence analysis and genetic association

DNA sequences of the 3426 bp assembled in healthy and mastitis buffaloes' segments were analyzed for polymorphisms using visual identification SNP analysis. Twenty-one SNPs were found based on the ATG start codon as position 1. Three SNPs in 5'UTR, five SNPs in exon1, one SNP in intron1, and 12 SNPs in exon3 (Table 2). SNPs c1.27 (C/A) and c.87 (C/A) were non-mutual (only present in mastitis buffalo) while the other SNPs were mutual, i.e., present in both healthy and mastitis buffaloes at different percentages.

Genotypic frequency for each SNP and Statistical analysis of mutual SNPs were calculated using the corrected P value (Bonferroni) Table 2.

Association between SNPs and mastitis

Four SNPs were found to be associated with mastitis: one in 5′UTR c.1–27(C/A) and three in coding region; c.87 (C/A) in exon1, c.575 (A/G), and c.576 (T/G) in exon3. The non-mutual SNPs c.1–27(C/A) and c.87 (C/A) were 100% CC homozygous in healthy buffaloes while in mastitis buffaloes they were 51%, CC, 41% CA, and 8% AA. The genotypic frequencies of the two mutual SNPs c.575 and c.576 (sharing amino acid 192) were statistically significant after Hardy–Weinberg equilibrium. The association of these two SNPs and susceptibility to mastitis was assessed by correcting the P values using Bonferroni adjustment (Table 2) and odd ratio (Table 3).

Buffaloes with dominant genotypes AA at c.575 and TT at c.576 were associated with mastitis resistance (OR < 1.00). Whereas those with recessive genotype GG at c.575 were associated with mastitis susceptibility (OR > 1.00).

To find out what is the impact of SNPs in the coding region, we investigated the protein using Protein prediction analysis.

Protein prediction analysis

TLR4 signal peptide

Analysis of the deduced polypeptide predicted the cleavage site of TLR4 protein between amino acid positions 25 and 26 with Likelihood 0.9722 as figured out using the SignalP 5.0 server showing that the mature protein is secretory and starts at position twenty-six.

Architecture domains of TLR4 protein

TLR4 protein domains were predicted using SMART analysis. They included low compositional complexity region (LC) 307–327; a trans-membrane domain (TM): 635–657; LRR C terminal (LRR-CT): 579- to 629 and TIR

Table 2 TLR4 SNPs in healthy and mastitis river buffalo, Egyptian breed

SNP position		Amino acids variation	Genotype frequency (%)			Corrected P value (Bonferroni)
c.1-g.150		–	CC	CG	GG	0.6103
	Healthy		54	39	7	
	Diseased		47	43	10	
c.1-g.149		–	AA	AG	GG	1.000
	Healthy		37	48	15	
	Diseased		37	48	15	
c.1-g.27[a]		–	**CC**	**CA**	**AA**	–
	Healthy		**100**	**0**	**0**	
	Diseased		**69**	**28**	**3**	
c.44		^{Thr} 15 ^{Met}	CC	CT	TT	0.0658
	Healthy		37	48	15	
	Diseased		60	35	5	
c.66		^{Arg} 22 ^{Ser}	AA	AT	TT	0.0658
	Healthy		37	48	15	
	Diseased		60	35	5	
c.87[a]		**^{Cys}29$^{stop/}$**	**CC**	**CA**	**AA**	–
	Healthy		**100**	**-**	**-**	
	Diseased		51	41	8	
c.89		^{Val} 30 ^{Gly}	TT	TG	GG	0.07233
	Healthy		86	12	2	
	Diseased		67	29	4	
c.90			AA	AG	GG	0.07233
	Healthy		86	12	2	
	Diseased		67	29	4	
c.94-g.41		–	TT	TG	GG	1.000
	Healthy		30	50	20	
	Diseased		30	50	20	
c.572		^{Ser} 191 ^{Tyr}	CC	CA	AA	0.08152
	Healthy		33	49	18	
	Diseased		17	49	34	
c.575		**^{His} 192 $^{Arg/Gln}$**	**AA**	**AG**	**GG**	**0.006156***
	Healthy		**60**	**35**	**5**	
	Diseased		**34**	**49**	**17**	
c.576			**TT**	**TG**	**GG**	**0.001653***
	Healthy		**40**	**47**	**13**	
	Diseased		**17**	**49**	**34**	
c.577		^{Lys} 193 ^{Glu}	AA	AG	GG	0.14995
	Healthy		27	50	23	
	Diseased		17	49	34	
c.579			GG	GA	AA	0.14995
	Healthy		27	50	23	
	Diseased		17	49	34	
c.647		^{Gly}216^{Asp}	GG	GA	AA	0.14995
	Healthy		27	50	23	
	Diseased		17	49	34	
c.662		^{Gly} 221 ^{Asp}	GG	AG	AA	0.09156
	Healthy		30	49	21	
	Diseased		17	49	34	

Table 2 (continued)

SNP position		Amino acids variation	Genotype frequency (%)			Corrected P value (Bonferroni)
c.672		^{Lys} 224 ^{Asn}	AA	AC	CC	0.09156
	Healthy		30	49	21	
	Diseased		17	49	34	
c.812		^{Ile} 271 ^{Arg}	TT	TG	GG	0.09156
	Healthy		30	49	21	
	Diseased		17	49	34	
c.816		^{leu} 272 ^{Phe}	CC	CA	AA	0.09156
	Healthy		30	49	21	
	Diseased		17	49	34	
c.2464		^{Gln} 822 $^{Lys/Pro/Thr}$	CC	CA	AA	0.61034
	Healthy		36	48	16	
	Diseased		30	49	21	
c.2465			AA	AC	AA	0.61034
	Healthy		36	48	16	
	Diseased		30	49	21	

SNPs positions were calculated taking the ATG start codon as position 1 based on the sequences

* Significant SNPs: in bold. a: No analysis was performed for non-mutual SNPs (present only in mastitis buffaloes)

Table 3 Association of genotype and mastitis

SNPs position	Genotype	P*	OR	Susceptibility
c.575 (A > G)	AA	0.0003*	0.3434*	Resistance
	AG	0.0517	1.7843	
	GG	0.0104*	3.8916*	Susceptible
c.576 (T > G)	TT	0.0004*	0.3072*	Resistance
	TG	0.7771	1.0834	
	GG	0.3638	1.3956	

*$P < 0.05$ = statistically significant; OR > 1.00 = buffalo susceptible to mastitis; OR < 1.00 = buffalo resistant to mastitis

domain: 674–819. This is in addition to 13 LRRs located at positions: 77–100, 101–124, 149–173, 174–197, 201–225, 372–393, 398–420, 421–442, 446–469, 470–494, 495–518 (LRR_TYP), 519–542 and 543–566 clustered toward the C-terminal (Fig. 1). The amino acid 192 (carrying two significant SNPs, c.575 and c.576) was present in the LRR4 domain (174–197).

Putative binding sites

From the 5′UTR flanking region investigated for putative binding sites three nucleotide mutations were detected:

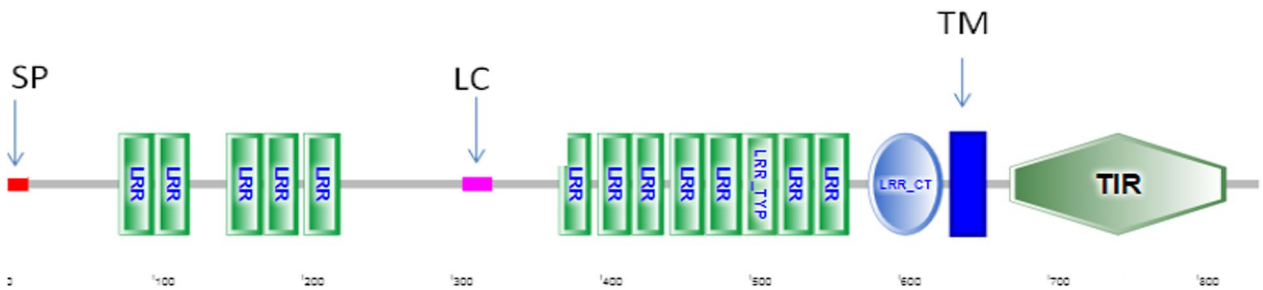

Fig. 1 Protein domain architecture of TLR4 gene in Egyptian Buffalo. Protein domain architecture of TLR4 gene in Egyptian Buffalo using SMART analysis. The red box stands for Signal peptide (SP); Pink box represents Low complexity regions (LC); Leucine-rich repeats: LRR; LRR_TYP: Leucine-rich repeats, typical; LRR_CT: Leucine-rich repeats C-terminal; blue rectangle stands for the transmembrane domain (TM); TIR: Toll/Interleukin-1 domain

SNP c.1-g.27 (C/A) (significantly associated with mastitis) was found in Chorion Factor (CF) whereas c1.-g.149 (G/A) and c1.-g.150 (C/G) were in Stress-respond element (STRE).

Discussion

Mastitis, a multi-etiological complex disease, is the most expensive production disease inflicting major economic losses to the dairy industry worldwide, especially in developed countries (Seegers et al. 2003). It affects animal health, the quality of milk, and the economics of milk production, causing huge financial losses (Sharma 2007). TLR4 plays a role in bovine mastitis; its association was examined in bovine by Gupta et al. 2015; Wang et al. 2007; Sharma et al. 2006; Li et al. 2014; Gulhane and Sanguan 2012; Arif et al. 2015. Mutations in the TLR4 gene were also investigated in Italian Mediterranean buffalo (Alfano et al. 2014), the Murrah breed (Sonawane et al. 2018), and six Indian buffalo breeds (Tantia et al. 2012). Eight out of 21 SNPs of the TLR4 reported in this study (three in 5'region: c.1-g.150, c.1-g.149 and c.1-g.27), four in exon1 (c.66, c.87, c.89 and c.90) and one in intron 1(c.94-g.41) were only present in Egyptian buffalo. No SNPs were reported in TLR4 exon2 of river buffalo breeds except in the study by Tantia et al (2012) who reported a single SNP at position 1341nt. These Genetic variations between breeds of buffaloes can be attributed to environmental and/or genetic factors.

The relatively high frequency of non-synonymous SNPs in TLR4 detected in the present study was also reported in 11 different cattle breeds (White et al. 2003); in Barki sheep (Sallam 2021), and Murrah buffalo (Roldan-Montes et al. 2020) is justified since the TLR4 gene is a cell-surface pattern-recognition receptor important to recognition of a broad of PAMPs.

It is worth mentioning that the reported three SNPs in the putative binding sites of TLR4 promotor region SNP c.1-g.27, c1.-g.149 and c1.-g.150 have been previously reported in the bovine promoter of *TLR4* genes (Sharma et al. 2006; Wang et al. 2009; Mishra et al.. 2018) and human and mouse TLR4 gene (Rehli et al. 2000). The variation in TLR4 promoter sequence of Egyptian buffalo breed might potentially influence the innate immune response against mastitis. Mishra et al. (2018) reported that nucleotide variability in TLR4 promoter region in cattle can alter Transcription factor binding sites.

SNPs association with mastitis

The present study revealed that four SNPs (c.1-g27, c.87, c.575, and c.576) were associated with mastitis. The two significant nucleotide variations c1-g27(C > A) and c.87 (C > A) occurred only in mastitis buffaloes at 3% and 8%, respectively, which may indicate that buffalo with CC genotype at both locations are resistant to mastitis. The SNP c.87 (P29) introduces a premature stop codon in mastitis buffaloes, beyond the signaling domain causing abnormally shortened TLR4 protein which may attribute to the loss of function in mastitis buffalo (Wang et al. 2009). Premature stop codon in the signaling domain may lead to the absence of functional TLR4 protein as reported by Roldan-Montes et al. (2020). It is worth mentioning that two nsSNPs (c.44 and c.66) reported in this study, although not statistically significant, were in the signal peptide domain which may affect directing the synthesized protein toward the secretory pathway. This calls for investigating larger numbers of buffaloes.

The two statistically significant nsSNPs c.575 (A > G) and c.576 (T > G), sharing codon 192, increase the number of possible nucleotide combinations in the codon and contributes to the protein variability since they can translate to three different amino acids His192Arg/Gln in the coded protein. The analysis revealed that the dominant genotypes AA at c.575 and TT at c.576 were associated with mastitis resistance, while recessive genotype GG at c.575 was associated with mastitis susceptibility. In other words, buffalo with histidine were likely resistant to mastitis whereas buffalo with arginine were more susceptible to mastitis.

The mutations in amino acid 192 in the LRR4 domain (p.174–p.197), part of the Putative coreceptors-binding region1 (CRBR1) which covers amino acids 24 to 273 in TLR4 protein of bovine (White et al. 2003), The mutations in amino acid 192 may affect the role of ligand recognition involved in the recognition of the lipopolysaccharide (LPS) of Gram-negative bacteria (Uematsu and Akira 2008 and Lu et al. 2008). CRBR1 serves as recognizing a broad of Pathogen-associated molecular patterns PAMP) from pathogens (Bannerman et al. 2002). It is worth mentioning that ten SNPs detected in this study, although not statistically associated with mastitis, fall in the CRBR1 region too.

The relatively high frequency of TLR4 non-synonymous SNPs detected in the present study and reported in 11 different cattle breeds (White et al. 2003); in Barki sheep (Sallam 2021), and Murrah buffalo (Roldan-Montes et al. 2020) is justified since the TLR4 gene is a cell-surface pattern-recognition receptor important to recognition of a broad of PAMPs.

Based on the four significant SNPs; c.1-g.27C > A and c.87C > A, c.575 A/G, and c.576 T/G, detected in this study; we may conclude that buffaloes that had the joint genotypes CCAT are more likely to be resistant to mastitis and those with genotype AAGG were susceptible with mastitis. This indicates that those genotypes have the potential to be molecular markers for assisted buffalo selection to improve milk production. A thorough

investigation of the bovine TLR4 gene is of immense value for increasing animal productivity.

Conclusions

The present study confirms the relation between TLR4 genotypes and mastitis resistance or mastitis susceptibility in river buffalo. The study suggested four SNPs (c1-g27C > A, c.87 C > A, c.575A > G, and c. 576 T > G) have the potential to be markers for assisted buffalo selection to improve milk production.

Abbreviations

SNPs	Single nucleotide polymorphism
SCC	Somatic cell count
SCS	Somatic cells scores
CMT	California mastitis test
TLR4	Toll-like receptor 4
SP	Signal peptide
LRR	Leucine-rich repeat domain
TM	A transmembrane domain
TIR	An intracellular Toll-interleukin-1 receptor domain
LC	Low complexity regions
OR	Odds Ratio test

Acknowledgements

Not applicable.

Author contributions

AA and SN designed, supported, analyzed the sequence, supervised the study, and wrote the manuscript. NO participated in the experiment and conducted the statistical analysis. ES and NS conducted the experiment. All authors have read and approved the manuscript.

Funding

This study was funded by the National Research Center, Egypt, Project No. 11020106 (2016–2019).The NRC administered the project through which the research was conducted. The fund covered the required supplies.

Availability of data and materials

We declare that all data generated and analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 5 April 2023 Accepted: 5 June 2023
Published online: 17 July 2023

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