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REVIEW ARTICLE
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An overview on single nucleotide polymorphism studies in mastitis research

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Abstract

Mastitis is an inflammatory condition of the mammary gland caused by microorganisms as diverse as bacteria, viruses, mycoplasma, yeasts and algae. Mastitis is an economically devastating disease mainly affecting the crossbred cattle in India. Control strategies against mastitis includes antibiotic therapy, vaccination, improvements in dairy cattle husbandry, farm and feeding management etc. but has met with little success.. Mastitis tolerance/susceptibility is difficult to measure directly and hence milk somatic cell count (SCC) or milk somatic cell score (SCS) is used as an indicator trait for mastitis as both traits are highly positively correlated. Single nucleotide polymorphism (SNP) marker is a single base change in a DNA sequence at a given position. SNP markers are the most preferred genetic markers nowadays. Currently most researches worldwide have been targeting molecular high density SNP markers that are linked to mastitis tolerance in an attempt to incorporate to understand the genetics of host resistance to mastitis and this knowledge will be helpful in formulating breeding programmes in an attempt to control mastitis. This article reviews various SNPs which are reported to be significantly associated with mastitis tolerance/susceptibility.

Keywords: mastitis, single nucleotide polymorphism, somatic cell count.

Introduction

Mastitis is an inflammatory condition of the mammary gland caused by microorganisms as diverse as bacteria, viruses, mycoplasma, yeasts and algae (give referance). The organisms that account for most of the cases are *Escherichia coli, Staphylococcus aureus, Streptococcus dysgalactiae, Klebsiella pneumoniae*, *Streptococcus agalactiae, Streptococcus uberis* and *Streptococcus bovis*. Other organisms like *Corynebacterium bovis, Klebsiella oxytoca*, *Pseudomonas aeruginosa, Nocardia, Pasteurella* spp., *Proteus* spp. *Mycoplasma* spp., *Brucella abortus, Trueperella pyogenes, Prototheca zopfii, Prototheca wickerhamii* and yeast are also involved [1]. The *Enterobacteriacae* accounts for 40.9 % of all mastitis cases, and *S. aureus, S. dysgalactiae, S. agalactiae* accounted for only 10% of clinical cases [2].

Mastitis can be manifested as clinical and subclinical forms. The clinical mastitis shows observable symptoms such as red and swollen mammary glands i.e., red swollen udder and production of clotted milk while subclinical mastitis do not reveal any apparent signs but is characterized by high somatic cell count (SCC), a normal or elevated body temperature, and milk samples that should test positive on culture. In India, subclinical mastitis was found more important (varying from 10-50% in cows and 5-20% in buffaloes) than clinical mastitis (1- 10%) [1]. The Indian dairy sector is facing nearly Rs. 60,000 million annual losses due to mastitis. The loss of ~ Rs. 4, 4000 million is due to subclinical mastitis is more than that of clinical mastitis i.e., ~ Rs. 1, 7000 million per annum [3]. The incidence rate of clinical mastitis was 25-60% worldwide [4] while in India usually had a higher rate. Control strategy against mastitis mainly relies upon antibiotic therapy, vaccination, farm management practices to limit the duration of infection and to contain infectious spread of pathogens through the herd. But these methods have met with little success. As a result of these control measures opportunistic pathogens such as Escherichia coli and Streptococcus uberis induced mastitis has become the leading cause worldwide [5]. As there are flaws in the current control strategies like vaccination being unsuccessful, antibiotic theory not only costs the farmer much but also making milk unfit for human consumption.

Hence there is a need to develop sustainable breeding strategies reduce incidence of mastitis and also to improve the quality of milk production [6]. Conventional breeding strategies based on quantitative genetics against mastitis are hampered as mastitis tolerance/susceptibility is a threshold trait and also due to low

additive genetic variances and heritability for health disorders [7]. A selection strategy by improving the host genetics through genetic marker selective breeding has now been practised widely [8]. Genomic selection utilises molecular high density single nucleotide polymorphism (SNP) markers [9]. A suitable control strategy can be devised only if we understand the host genetics and judicious application of the newer techno-logies like SNPs to plan better breeding policies that will incorporate traits like mastitis tolerance. In India, the dairy breeding programmes are selected solely for high milk production with little emphasis have been given on disease resistant traits. Nevertheless, breeding goals of many countries has been diversified to include health and functional traits in an effort to minimize and reverse the decline in these traits [10]. In order to incorporate health traits like mastitis tolerance it is essential to have basic knowledge on the existence of genetic variation in Indian cattle population particularly the crossbred which are more susceptible to the disease. This article will review the various SNP markers that are found to be associated with mastitis tolerance/susceptibility trait.

Somatic cell count (SCC)

Somatic cell count is the gold standard to measure mastitis [11] and is used as an indicator of udder health [12]. SCC is related to the immunological status of the udder and increases in response to an inflammatory stimulus like bacterial infection [13, 14]. Somatic cells are a part of the natural defence mechanism which include lymphocytes, macrophages, polymorph nuclear cells and some epithelial cells [15].

SCC or somatic cell score (SCS) is usually used as the indicator for mastitis as data on clinical mastitis are difficult to acquire and are costly. SCS and mastitis are genetically correlated and the correlation being on an average 0.7 [16] and ranges from 0.55 to 0.93 [17]. The high positive correlation suggests that both SCC and mastitis occurrence are partly caused by the expression of the same trait [18]. Various studies have confirmed that SCC or SCS is the most suitable single trait for the reduction in incidence of mastitis through indirect selection [16]. One of the advantages of using SCC is that the data is available easily but is never a clear substitute to measuring mastitis directly as genetic correlation is not perfect.

Single nucleotide polymorphism (SNP)

SNP marker is a single base change in a DNA sequence usually of two possible nucleotides at a given position [19]. SNPs have many advantages over other sorts of polymorphism in the genetic dissection of complex traits and diseases, and for population-based gene identification studies like their abundance, being found throughout the genome, i.e., in exons, introns, inter-genic regions, in promoters or enhancers, [20]. SNP in coding region may directly impact a relevant protein, an intronic SNP can influence splicing [21], and a SNP in a promoter can influence gene expression [22]. Alleles of neighbouring SNPs that are in linkage disequilibrium can be exploited in genetic linkage and direct association studies [23]. Microarray and other high throughput technologies can be efficiently utilised for genotyping of hundreds or thousands of SNPs [24]. Mutation rates are lesser in SNPs than other forms of polymorphisms [25, 26].

There are various methods that are currently available for genotyping SNPs like PCR-RFLP, which is done if the SNP to be studied involves a restriction enzyme (RE) site. PCR products are digested with restriction enzyme and are run on agarose gel electrophoresis. The genotypes can be identified based on the size differences of fragments produced by RE digestion. Allele-specific (AS) PCR approach utilises three PCR primers and separate reactions for wild and mutant allele has to be carried out. The primers for AS-PCR can be designed by tools like WASP (Web-based Allele-Specific PCR Primer designing tool) [27]. Another technique used for genotyping SNPs are Tetra Primer Amplification Refractory Mutation System (T-ARMS-PCR). This technique employs two primer pairs to amplify the two different alleles of a SNP in one single PCR reaction. The inner primers are so designed to have an allele-specific mismatch at 3′-terminal base and additional mismatch at position –2 from 3′-terminus. Primer design program for tetra primer ARMS-PCR is available through the internet at http://cedar.genetics.soton.ac.uk/public_html/primer1.html [28].

Also various sophisticated genotyping assays like TaqMan® SNP Genotyping Assays which rely on the 5'-3' exonuclease activity of Taq polymerase which is used to degrade an internal fluorescence resonance energy transfer (FRET) probe that contains a reporter and a quencher fluorescent dye. As long as they are linked to the oligonucleotide, the dyes are close together and the fluorescence is quenched. Upon degradation of the probe by the Taq polymerase, the fluorophore is released and the fluorescence thus emitted can be monitored. This is one of the most sensitive SNP genotyping assay but the limiting factor would be the cost involved. Various other techniques that is available for genotyping SNPs includes genetic bit analysis, molecular beacon, ligation, padlock probe, pyro sequencing etc. [29-31].

Single nucleotide polymorphisms associated with Mastitis

Single nucleotide polymorphisms are now the widely used genetic marker in the area of mastitis tolerance/susceptibility. Association studies of SNPs with mastitis tolerance/susceptibility have resulted in building a strong foundation on the niche of research on mastitis enabling new areas of focus in selection of animals. Studies on the single nucleotide polymorphisms in the bovine chemokine receptor type 2 (*CCR2*) gene and its association with health and production

traits in Canadian Holsteins using tetra primer ARMS-PCR has found that the allele substitution effect of the *CCR2* rs41257559:C>T SNP on SCS was significant [32]. In another study using tetra primer ARMS-PCR in bovine *CARD15*, four different polymorphisms have been identified *viz.*, c.2886-14A>G, c.3020A>T, c. 4500A>C and c.4950C>T [33].

When toll like receptor *4TLR4* gene was screened for its association with somatic cell score in dairy cattle, significant association between the SNPs and SCS was found. SCS of individuals with a CC genotype were found to be significantly lower than that of the TT genotype [34]. Investigation of SNPs in TLR4 gene is associated with subclinical mastitis in Holstein cows using TaqMan allelic discrimination had shown that animals that with combined genotypes AACCCC, GGTCGG and GACCGC had the lowest somatic cell scores. These genotypes were predicted to have the potential to be applied as molecular markers for assisted animal selection to improve milk quality [35].

Ogorevc *et al* [36] have combined information related to mammary gland from different published sources. These information regarding genetic markers for milk production traits and mastitis was integrated into a database of various candidate genes and their location on the chromosomes displayed as genetic map. They found that the highest density of mastitis related QTL were located on BTA3 and BTA14.The authors reviewed various candidate genes like *IL8RA*, *TLR4*, *BoLA-DRB3* etc. which were found to be associated with mastitis either by association studies or by expression studies. Forty four genes found by multiple independent analyses were suggested as the most promising candidate genes. These candidate genes were then analysed in silico for expression levels in lactating mammary gland, genetic variability and top biological functions in functional networks.

In a whole genome scan to map QTL for milk production traits and somatic cell score in Canadian Holstein bulls for ascertaining the genetic mechanism affecting milk production traits, SNPs were studied with regard to milk yield, protein yield, protein percentage, fat yield, fat percentage, somatic cell score and persistency of milk. This study has reported significant association of 11 SNPs, which are located on various genes with SCS. The SNPs which were found to have significant association were rs41637122, rs41628293, rs41601522, rs41576572, rs29014958, rs41578926, rs41654340, rs41650611, rs41606777, rs41648482 and rs41608052 [37].

Analysis of SNPs on lactoferrin gene with mastitis in Chinese Holstein cattle using PCR-RFLP found significant association between combined genotypes of three SNPs, haplotype and SCS [38]. In another study conducted by genotyping of SNPs by tetra primer ARMS-PCR, single nucleotide polymorphisms of *SPP1* gene has revealed that the SNP *SPP1*c.-1301G>A having an impact on EBV for SCS ($P < 0.001$) using statistical analysis and by using an allele substitution model, *SPP1*c.-430G>A, SPP1c.-1251C >T, and SPP1c.*40A>C were reported to have an impact on SCS. [18].

DNA sequence polymorphisms within the bovine guanine nucleotide-binding protein Gs subunit alpha (Gsa)-encoding (*GNAS*) genomic imprinting domain were found to be associated with performance traits. An SNP in the bovine *NESP55*gene (rs41694656) was found to be associated with somatic cell count ($P \leq 0.01$) [39]. Analysis the SNPs of *CACNA2D1* gene and its association with milk somatic cell score by PCR-RFLP method found significant association between A526745G and somatic cell score. They also found that the mean of genotype GG was significantly lower than those of genotype AG and AA ($p=0.0469$) [16].

SNPs on the *MBL1*gene were found to be associated with milk performance traits in Chinese native cattle. Statistical analyses has revealed significant association between g.2651G>A and SCS which suggested a possible role of this SNP in the host response against mastitis. Analysis of combined genotypes identified GGC/AAC with the lowest are SCS favourable combinations for mastitis resistance [8]. *MBL1* gene possibly contributes to bacterial infection resistance and was projected as a molecular marker of milk production traits to control mastitis [40]. The *MBL1* polymorphisms were indicated as indirect marker to improve mastitis resistance trait in cattle [41]. Correlation between SNPs of *MBL2* and somatic cell score suggested possible roles of the SNPs in the host response against mastitis [42]. Identification of SNPs in the bovine Toll-like receptor 1 (*boTLR1*) gene with its association with health traits in cattle have concluded that animals with the GG genotype (from the tag SNP -79 T > G) had significantly lower *boTLR1* expression in milk somatic cells when compared with TT or TG animals [43].

Association study between SNP and SCS in bovine breast cancer 1 (*BRCA1*) gene through DNA sequencing, PCR-RFLP and created restriction site PCR (CRS-PCR) methods has found three SNPs (G22231T, T25025A, C28300A) and 24 combinations of these SNPs. When the authors studied their association with SCS, C28300A was found to be significantly associated [44]. In another study involving *BRCA1*, 51 SNPs were screened in which three SNPs (c.5682 G>C, c.26198 C>T and c.46126 G>T) were genotyped by PCR-RFLP and CRS-PCR methods. A significant association with SCS was found in the SNP c.46126 G>T. The results of combined genotypes analysis of three SNPs showed that HHLLNN genotype were having the highest SCS were easy target for the mastitis while GGKKMM genotype with the lowest SCS was favourable for the mastitis resistance [45].

Search of single-nucleotide polymorphisms of complement component 4 gene (*C4A*) in Chinese Holstein cattle was found to be associated with milk performance traits. The statistical analyses revealed that cows with rs132741478: g.2994 A > G-AG and

Table-1: Various single nucleotide polymorphisms that are found to be significantly associated with mastitis tolerance

SNP	Gene	Reference
rs41637122	*APP*	[37]
rs41628293	*BFSP1*	[37]
c.46126G>T	*BRCA1*	[44]
C28300A	*BRCA1*	[44]
rs132741478	*C4A*	[46]
rs137485678	*C4A*	[46]
A526745G	*CACNA2D1*	[16]
rs43710287	*CARD15*	[33]
rs43710288	*CARD15*	[33]
rs43710289	*CARD15*	[33]
rs43710290	*CARD15*	[33]
rs41257559	*CCR2*	[32]
rs41648482	*CHS1*	[37]
rs41650611	*DNTT*	[37]
rs41694656	*GNAS*	[39]
rs41654340	*HIST1H2BK*	[37]
g.4432T>C	*Lf*	[38]
g.3879_3880insG	*Lf*	[38]
rs29014958	*LOC510933*	[37]
rs41608052	*LOC517432*	[37]
rs110326717	*MBL1*	[8]
rs41601522	*MGC142355*	[37]
rs41576572	*MGC142850*	[37]
rs41606777	*SLC18A2*	[37]
c.1301G>A	*SPP1*	[18]
g.9788C>T	*TLR4*	[34]

rs137485678: g.3649 G>C-CC have significantly lower somatic cell scores (SCS, P<0.01). The results confirmed that rs132741478: g.2994 A>G in the coding sequence of the β-chain of the bovine *C4A* gene is related to mastitis resistance [46]. Single nucleotide polymorphism located on heat shock factor (*HSF1*) was found to be associated with mastitis [47]. Selective genotyping in combination with logistic regression analyses revealed polymorphisms of SNPs on *TLR4* and on *CACNA2D1* being associated with desirable effects on clinical mastitis [48].

In a study on SNPs of Bovine peptidoglycan recognition protein 1 (*PGLYRP-1*) has identified a total of ten SNP loci. The association study has shown that T-35A, T-12G and G-102C to be significantly associated (P<0.05) with somatic cell score (SCS) [49]. SNP mining in BTA6 (*FAM13A, ABCG2, OPN, LAP3, HCAP-G, PPARGC1A*) and somatic cell count (SCC) in milk identified different genotypes by the PCR-RFLP method and the results showed statistically significant differences between mean values of SCC in analysed cows with different genotypes of *FAM13A1 G85A* and combined genotypes *OPN* and *FAM13A1* [50]. Analysis of the SNPs of Protein arginine N-methyltransferase 2 (*PRMT2*) gene and mastitis has identified four SNPs viz., g. C10202T, g.A10276G, g. C24375T and g.C24385T using PCR-RFLP technique. [51].

Association study of SNP at position +777 within the *CXCR1* gene and SNP at position -1768 (rs41255711) and somatic cell score in Holstein dairy cattle could not reveal any significant statistical associations clearly contradicting previously published studies [52]. In another study evaluating three single nucleotide polymorphisms contained in Toll-like receptor 4 (*TLR4*) and lactoferrin (*LF*) genes associated with mastitis traits: *TLR4* P-226, *TLR4* 2021, and *LF* P-28 were genotyped using PCR-RFLP and high-resolution melting quantitative PCR. The TT genotype of *TLR4* 2021 had no associations with somatic cell score [53]. *SLC11A1* protein is involved in bacterial killing and is regarded as candidate gene for bovine mastitis resistance. An SNP in coding region of *SLC11A1* had resulted in an amino acid variation of p.P356A but no significant associations with mastitis were found [54]. These studies showed that the associations between SN60Ps and mastitis are population specific and vary between populations. Hence it is needed to individually screen and verify the presence of SNPs linked with mastitis in different populations. Some of the single nucleotide polymorphisms which are reported to be associated with SCS have been summarized in Table-1.

Conclusion

Mastitis has come down heavily on the economics of dairy farming worldwide making a greater focus in controlling this disease. Identification of various single nucleotide polymorphisms associated with mastitis is very promising in devising a suitable control strategy in combating mastitis. One of the limiting factors would be that, though an SNP found to be associated with mastitis in a particular population would not guarantee that such an association exists in another population. Hence it is necessary to ascertain the association of SNPs with mastitis in various populations and to pick out those which are linked with quantitative trait loci. Single nucleotide polymorphisms studies in relation to mastitis would enhance the niche area of mastitis research and thereby helps in devising a suitable control strategy against this economically important disease.

Authors' contributions

VNMA prepared the initial version of the manuscript. VNMA, AK, AR, RS, VM and PD assisted in literature collection. VNMA and MP drafted and revised the manuscript for critical scientific corrections. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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