Original Article

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PEPTIDOGLYCAN RECOGNITION PROTEIN-1 GENE POLYMORPHISM AND ITS ASSOCIATION WITH MASTITIS IN MURRAH BUFFALO

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ABSTRACT

A wide variety of environmental or contagious microorganisms implicated in mastitis, impede the economic growth of dairy sector. Identification of polymorphism in candidate gene of host's immune system and to rule out mastitis resistant allelic form of candidate gene usually remains prime focal point of research. Bovine peptidoglycan recognition protein-1 (PGLYRP-1), exclusively present in the granules of polymorphonuclear leukocytes has direct microbicidal properties. The present study was carried out to find the association between PGLYRP-1 polymorphic alleles with mastitis. Milk samples for somatic cell count and blood samples for PCR-RFLP analysis of PGLYRP-1 gene were collected from 20 mastitis negative and 20 mastitis positive Murrah buffaloes. There was significant difference in somatic cell count of mastitis and mastitis free animals. All amplified PCR products of ~862 bp size of partial region of PGLYRP-1 gene were subjected to each restriction enzyme (HincII or TaqαI or ApaI). Polymorphism in the partial region of PGLYRP-1 gene had not been established using PCR-RFLP as uniformity in pattern of digested fragments was seen. Target sequence PGLYRP-1 gene of Murrah buffalo was cloned and sequenced. BLAST analysis revealed sequence identity of PGLYRP-1 of Murrah buffalo with *Bos taurus* (JN085441.1) sequence at NCBI was 96%, 96% with *Bos indicus* (JN085440.1) and 96% with *Bos indicus* X *Bos taurus* (EU746454.1). In phylogenetic tree, the target sequence of PGLYRP-1 gene of *Bubalus bubalis* are found more closely related to *Bos taurus* than to *Bos indicus.*

Keywords: *Bubalus bubalis*, buffaloes, bovine, mastitis, peptidoglycan recognition protein-1, polymorphism, PCR-RFLP

INTRODUCTION

Murrah is well known breed for its milk production among buffalo breeds. Although milk yield can be augmented drastically *via* inclusion of production traits in breeding policies, however, breeding policies remain ineffective without

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inclusion of disease resistant traits into the herd as higher milk yielders are more prone to mastitis, major peril to dairy industry (Oltenacu and Broom, 2010). The characteristics of the etiological agent, the genetics and physiological state of the animal determine susceptibility or resistance towards the disease. Exposure of antibiotic resistant pathogen, mismanagement, immune-compromised status of host, inferior quality, and intensity of immune response to encounter pathogen escalates mastitis cases.

Mastitis is a multi-factorial disease, and animals provide discrepancy response to various infectious challenges due to variation in genetic make-up of animals despite same management conditions. Consequently, many research groups have attempted to identify candidate genes polymorphism associated with mastitis resistance (Ogorevc *et al*., 2009). Genes involved in innate immunity are suitable candidates to explore mastitis resistant polymorphism. Strategic selection of animals having mastitis resistant trait and the incorporation of this trait into the herds is one promising approach to combat mastitis. Mastitis resistance trait is complex, and genes involved in the immune response are considered as strong candidates for this trait (Oviedo-Boyso *et al*., 2007).

PGLYRP1, an innate immunity protein, plays an imperative role both in antibacterial defenses and several inflammatory diseases (Liu *et al*., 2013). Mammalian PGLYRPs are differentially expressed in various organs/ tissues such as bone marrow, granules of polymorphonuclear (PMNs) leukocytes etc. and have two major functions: amidase activity and antibacterial activity (Gelius *et al*., 2003, Kang *et al*., 1998, Liu C *et al*., 2001).

Bovine PGLYRP-1 has direct microbicidal activity for many Gram-positive bacteria, Gram-negative bacteria, and fungus (Tydell *et al*., 2003). Bovine PGLYRP1 gene includes 3 exons and spans about 1965 bp of genomic DNA. The global scenario of *Mycobacterium avium paratuberculosis* (MAP) infection in animal recently has been concluded (Agrawal *et al*., 2019). Pant *et.al.* (2011) identified three single nucleotide polymorphisms (SNPs) in the gene encoding bovine peptidoglycan recognition protein 1 and assessed all SNPs association with susceptibility of *Mycobacterium avium paratuberculosis* (MAP) infection in dairy cattle. The association of one synonymous SNP c.480G>A with the MAP infection was marginal established.

Elevated level of milk somatic cell count (SCC) having >95% of polymorphonuclear (PMNs) as a consequence of encounter of mastitis pathogen are indicators of the inflammatory response in order to combat the infection. However, excessive inflammation is detrimental to udder health. Breed-wise and species-wise variations in the pattern of immune response despite same stimulant dose divulge resistivity of breed/species towards mastitis pathogen (Sulab *et al*., 2018; Sulab *et al*., 2019). The SCC is an indicator of both the resistance and the susceptibility of cows to intra-mammary infection. Improving udder health through breeding can be based on indirect selection for low SCC. SCC is a more accurate measure of udder health than the records of clinical mastitis.

Breeders select the animal on the basis of performance or appearance of trait. This time-consuming complexity selection method of breeding insists marker assisted selection (MAS) or identification of better genotype at early age *via* DNA marker. MAS enables early selection of mastitis resistant animals before actual expression of the traits, leading to increased genetic progress within a very short time period. Due to advent of

molecular genetics techniques, DNA sequence and polymorphism of candidate gene (s) for economic and disease resistance traits are being considered as an aid to selection of the elite animals. Restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP) used to be a mean of finding gene polymorphism. If linkage disequilibrium between PGLYRP-1 gene and mastitis is established, MAS can be used for improving resistance to mastitis. Hence, the aim of present study was to study the polymorphism in PGLYRP-1 gene in Murrah buffaloes by using PCR-RFLP technique and to find the association between PGLYRP-1 polymorphic alleles with mastitis.

MATERIALS AND METHODS

Forty unrelated Murrah buffaloes (including 20 mastitis positive and 20 mastitis negative) from Govt. Livestock Farm, Hisar; Central Institute for Research on Buffaloes, Hisar; Buffalo Research Centre, College of Animal Sciences and TVCC, LUVAS, Hisar were taken in the experiment. Milk sample from all quarters of udder of each animal was collected for somatic cell counting (SCC) for ruling out the presence of sub-clinical mastitis. Blood (around 10 ml) from each animal was collected aseptically by jugular vein puncture in a sterile in 15 ml sterile polypropylene centrifuge tube containing 0.5 ml of 0.5M EDTA as anticoagulant. DNA was isolated from blood by phenol-chloroform isoamyl (PCI) method as described by Sambrook *et al.* (1989). The quality of extracted DNA was assessed by the ratio of absorbance at 260 and 280 nm ($A_{260/280}$ ratio).

Primer designing and Polymerase chain reaction (PCR)

PGLYRP-1 gene specific primers were designed using Primer BLAST software from NCBI as indicated in below and the primers were custom synthesized from Sigma (USA).

The optimal annealing temperature of PGLYRP-1 gene specific primers was standardized using extracted DNA from blood samples via Gradient PCR and reaction conditions of PCR were also standardized. For conventional PCR, PCR reaction mixer was prepared for each reaction (25 µl) by adding the following components in order: Nuclease free water (NFW) (17.75 µl); 10x Taq buffer (2.5 µl), dNTPs (0.5 µl), 20 pm/µl sense primers (0.5 µl), 20 pm/µl antisense primers (0.5 µl), 50 ng/µl gDNA template (3 µl) and 5 U/µl Taq DNA polymerase (0.25 µl). The PCR reactions were carried out in thermocycler (Eppendorf Master Cycler Gradient™) using thermal cyclic conditions as follows: initial denaturation at 95°C for 5 min, 35 cycles (denaturation, 94°C, 45 seconds; annealing, 65.1°C, 40 seconds; extension, 72°C, 1 minute), final extension at 72ºC for 5 minutes and held at 4ºC.

Restriction digestion of amplified DNA

For RFLP analysis, the reaction mixture for RE digestion was prepared in 0.2 ml tubes by adding the following component in order: NFW (2 µl), 10x Buffer (2 µl) and RE enzyme (TaqαI/ HincII /ApaI) (1 µl). After proper mixing, 15 µl of PCR product was added in each tube. All these steps were carried out on ice. The 20 µl reaction mix was kept for digestion in incubator at 37°C overnight. After the digestion, the enzyme was inactivated at 80°C for 20 minutes. and the RE products were stored at 4°C for further analysis.

Cloning of PCR products

The amplified PCR products were purified using QIAquick gel extraction kit (QIAGEN) for removing the primer dimer and non-specific amplified products prior to cloning and custom sequencing. The amplified PCR products of PGLYRP-1 and pJET 1.2/blunt cloning vector (CloneJET™ PCR cloning Kit, Fermentas) were digested with blunting enzyme before ligation. After enzymatic polishing, amplified products were ligated to pJET 1.2/blunt vector using T_4 DNA ligase. JM107 strain of *Escherichia coli* competent cells were prepared by the method described by Sambrook *et al.* (1989). The competent cells were transformed with the ligated mixture and transformants were spread on LB agar plates containing ampicillin (50 µg/ml) for selection. *E. coli* cells having recircularized pJET 1.2 cloning vector express a lethal restriction enzyme so it is not propagated. As a result, only *E. coli* cells having the recombinant vector containing the insert appear on culture plates. PGLYRP-1 gene target region specific primer was used to screen the colonies appeared on the plates to check the presence of insert. The bacterial cells from the colonies were suspended in 10 µl NFW and boiled for 10 minutes. This suspended solution was centrifuged at 9,440 x g for 5 minutes and 1 µl of supernatant was taken as DNA source for PCR. The PCR mixture and touch PCR conditions were kept same as described in conventional PCR. All the white picked colonies were propagated in LB-Amp broth and incubated in shaker incubator (REMI) at 150 rpm at 37ºC overnight (O/N). This bacterial suspension was used further for plasmid isolation *via* mini preparation method (alkaline lysis method) as described by Sambrook and Russell (2001) with minor modifications.

Automated nucleotide sequencing and nucleotide sequence analysis

The recombinant plasmids from clones positive by touch PCR were selected for sequencing. The vector specific primer (Pjet1.2 Forward/ Pjet 1.2 Reverse) were used for sequencing using automated DNA sequencer Applied Biosystem 3130 XL Genetic Analyzer at Department of Animal Biotechnology, College of Veterinary Sciences, LUVAS, Hisar. The sequences obtained from automated DNA sequencer were analyzed using NCBI BLASTn online software after converting sequences into FASTA format. (Website: http://www.ncbi.nlm.nih.gov). Clustalw2 programme was used for multiple sequence alignment of buffalo PGLYRP-1 gene sequence with other species DNA sequences retrieved from NCBI database. The phylogenetic tree was also constructed using MEGA4 software to show relatedness among the species with respect to PGLYRP-1 gene fragment.

RESULTS AND DISCUSSIONS

Somatic cell count (SCC) has been used to differentiate between mastitis infected and non-infected animals for the last three decades. The average SCC of collected milk samples from healthy animals was $0.40x10^5 \pm 0.4x10^4$ (i.e. below 1 lac) while average SCC in mastitis milk samples was $35.2x10^5 \pm 46x10^4$ (i.e. above 1 lac). These findings support the findings of Bytyqi *et al.* (2010). There was a significant ($P<0.05$) difference in SCC of healthy and mastitis animals.

Infectious diseases impede the development of the dairy sector and mastitis poses enormous economic losses to dairy industry. Apart from managemental care, superlative strategy to eliminate major obstacles in the sustainability of the dairy sector is to genetic selection of disease

resistant animals having superior quality traits and to incorporate disease resistant trait into herd. One of the most practical means for combating mastitis is to enhance the natural host defence mechanisms as vaccination remains ineffective due to involvement of multiple etiological agents (Detilleux *et al*., 1994). Although Mastitis resistant trait is complex. However, genes involved in the immune response have been considered as strong candidates for resistivity towards mastitis (Alluwaimi *et al*., 2003; Rambeaud *et al*., 2003; Oviedo-Boyso *et al*., 2007).

Bovine PGLYRP-1 binds to a variety of microbial components and kills various microorganisms. Consequently, bovine PGRPs play an imperative role for suppressing infectious diseases in bovine species (Tydell *et al*., 2006). However, information regarding the polymorphisms within the bubaline PGLYRP-1 gene is very scanty. Three SNPs in gene encoding bovine PGLYRP-1 were observed during MAP infection and one SNP was marginal associated with MAP susceptibility (Pant *et al*., 2011). Exploration of PGLYRP-1 gene polymorphism and association of polymorphic allele with resistivity towards mastitis pathogen may help in genetic basis of combating mastitis.

PCR-RFLP is one of the efficient tools to find genetic diversity in man and animals. In man, PCR-RFLPs have been used in the prenatal diagnosis of haemoglobinopathies (Little *et al*., 1980) and have also extended the utility of polymorphic markers in the detection of genes responsible, for other genetic disorders such as Retinitis pigmentosa (Bhattacharya *et al*., 1984), Duchenne muscular dystrophy (Murray *et al*., 1982), and Huntington's chorea (Gusella *et al*., 1983). PCR-RFLP is faster and more sensitive than traditional RFLP (Ivinson and Taylor, 1992). Hence, PCR-RFLP was used in the present study to explore PGLYRP-1 gene polymorphism during mastitis pathogen infection.

$A_{260/280}$ ratio of all extracted genomic DNA was in range of 1.7 to 1.9. The optimal annealing temperature of PGLYRP-1 gene specific primers was 65.1°C. The amplified PCR products of ~862 bp size of partial region of PGLYRP-1 gene were obtained as shown in Figure 1 (a,b). All amplified PCR products of PGLYRP-1 gene digested by HincII or TaqαI or ApaI RE showed monomorphic band patterns. After HincII RE digestion of each PCR amplified product, two different sizes of fragments (i.e. 572 bp and 290 bp) were obtained as shown in Figure 2(a,b). TaqαI RE digestion of each PCR amplified product yielded different sizes of fragments (i.e. 625 bp, 210 bp and 27 bp) as shown in Figure 3 (a,b) while ApaI RE digestion yielded two different sizes of fragments (i.e. 582 bp and 280 bp) as shown in Figure 4 (a, b). The association between PGLYRP-1 gene with mastitis could not be established as polymorphism in the partial region of PGLYRP-1 gene had not been observed using PCR-RFLP. This might have occurred due to the small sample size and small amplicon size. However, there was a significant

Table 1. Designation and sequence of primers for PCR assay targeting PGLYRP-1 gene.

Target gene	Primers	Oligonucleotide sequence (5'-3')	Product size (in bp)
PGLYRP-1 gene (Accession no. JN085441.1)	Sense	ATGGGGTTAGGGATGCGAAAAGGTG	862 bp
	Anti-sense	ACAGGGCTGGGGGATAAGATGAGTGA	

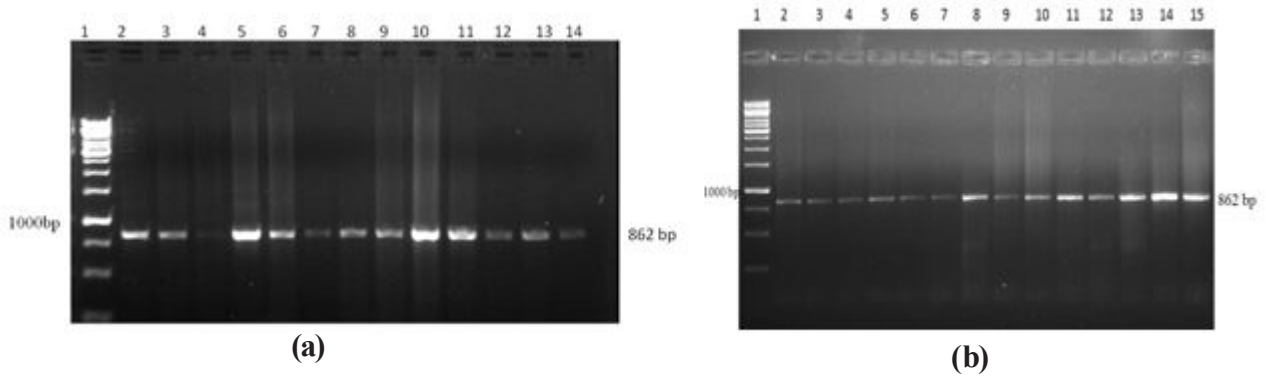


Figure 1. PCR amplification of PGLYRP-1 gene using extracted DNA from collected blood samples of mastitis free animals (a) and mastitis positive (b).
Lane 1: 1000 bp ladder; Lane 2 to 14/15, 862 bp amplified PCR product of PGLYRP-1 gene.

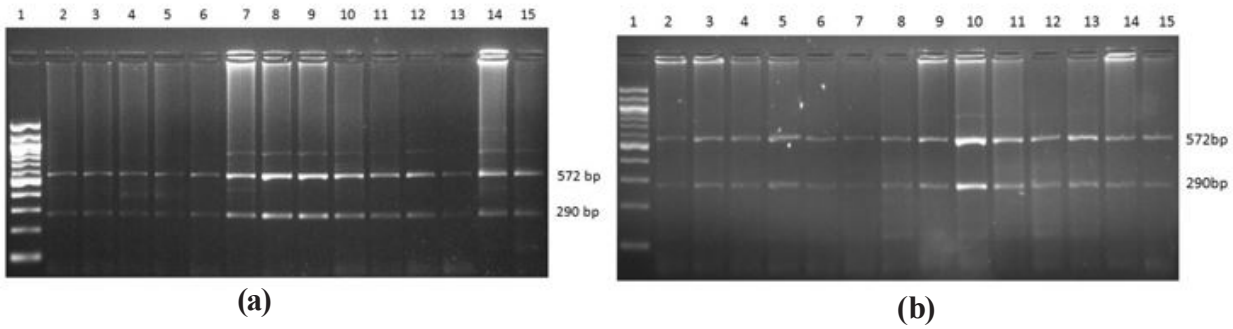


Figure 2. Electrophoretic mobility of fragments obtained by digestion of PGLYRP1 gene PCR product of mastitis free (a) and mastitis positive (b) Buffalo with Hinc II enzyme in 3% agarose gel.
Lane 1: 100 bp DNA ladder; Lane 2 to 15: digested fragments of sizes~ 572 bp and 290 bp.

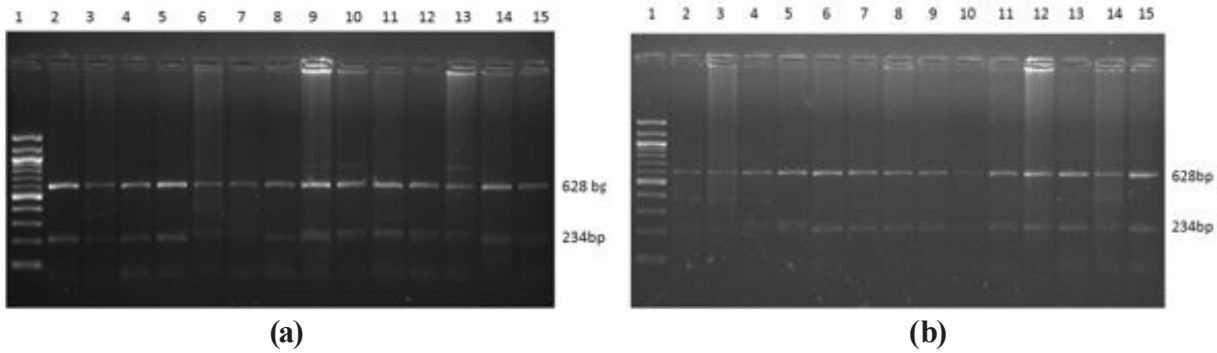


Figure 3. Electrophoretic mobility of fragments obtained by digestion of PGLYRP1 gene PCR product of mastitis free (a) and mastitis positive (b) Murrah buffalo with TaqαI enzyme in 3% agarose gel. Lane 1: 100 bp DNA ladder, Lane 2 to 15, digested fragments of sizes~ 628 bp and 234 bp.

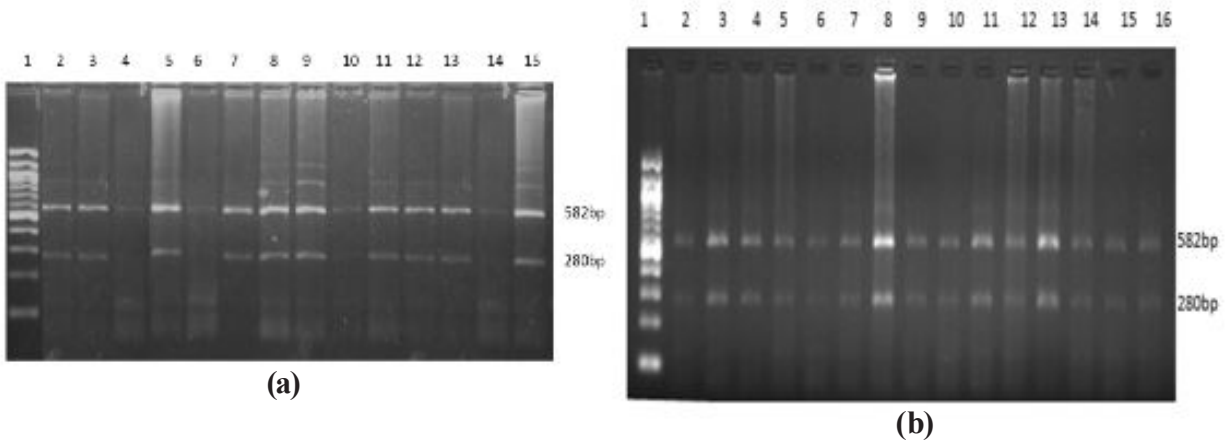
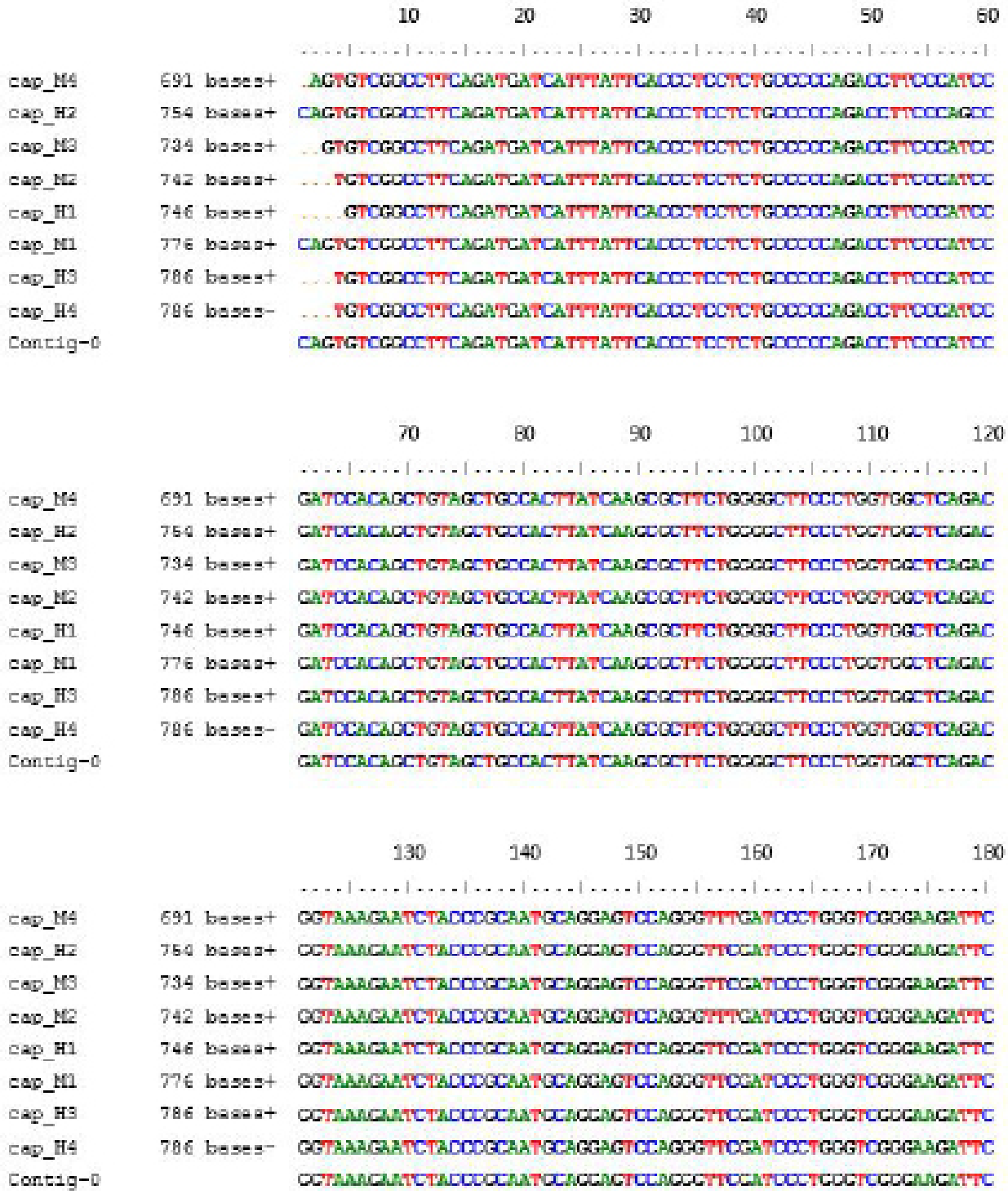


Figure 4. Electrophoretic mobility of fragments obtained by digestion of PGLYRP1 gene PCR product of mastitis free (a) and mastitis positive (b) Murrah buffalo with ApaI enzyme in 3% agarose gel. Lane 1: 100 bp DNA ladder; Lane 2 to 15/16, digested fragments of sizes~ 582 bp and 280 bp.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cap_H2 754 bases+
cap_M3 734 bases+
cap_M2 742 bases+
cap_H1 746 bases+
cap_M1 776 bases+
cap_H3 786 bases+
cap_H4 786 bases-
Contig-0
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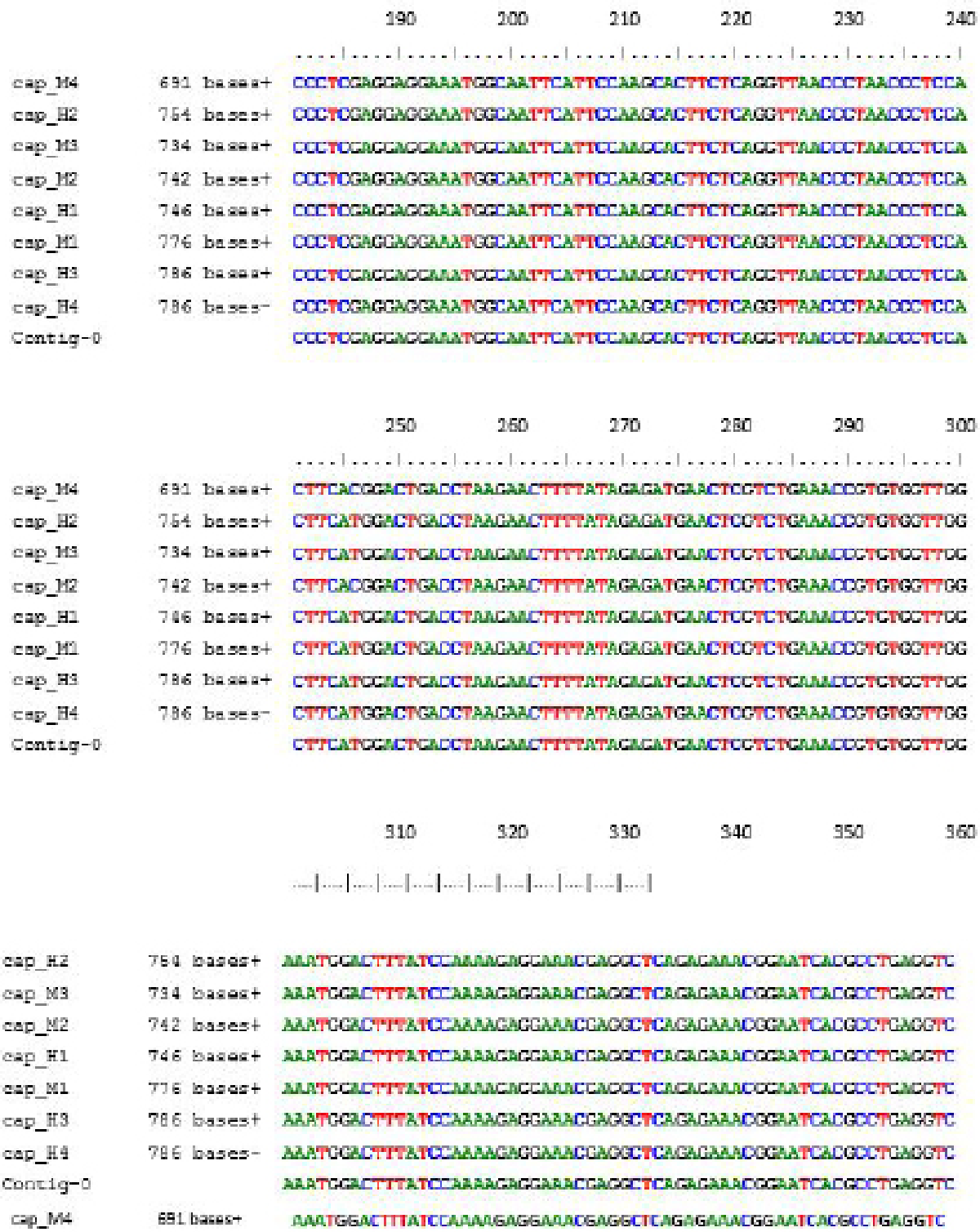
A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cap_M3 734 bases+ CCCTCGAGGAGGAAATGGCAATTCATTCCAAGCACTTCTCAGGTTAACCCTAACCCTCCA
cap_M2 742 bases+ CCCTCGAGGAGGAAATGGCAATTCATTCCAAGCACTTCTCAGGTTAACCCTAACCCTCCA
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cap_H4 786 bases- CTTCATGGACTGACCTAAGAACTTTTATAGAGATGAACTCCTCTGAAACCGTGTGCTTGG
Contig-0 CTTCATGGACTGACCTAAGAACTTTTATAGAGATGAACTCCTCTGAAACCGTGTGCTTGG
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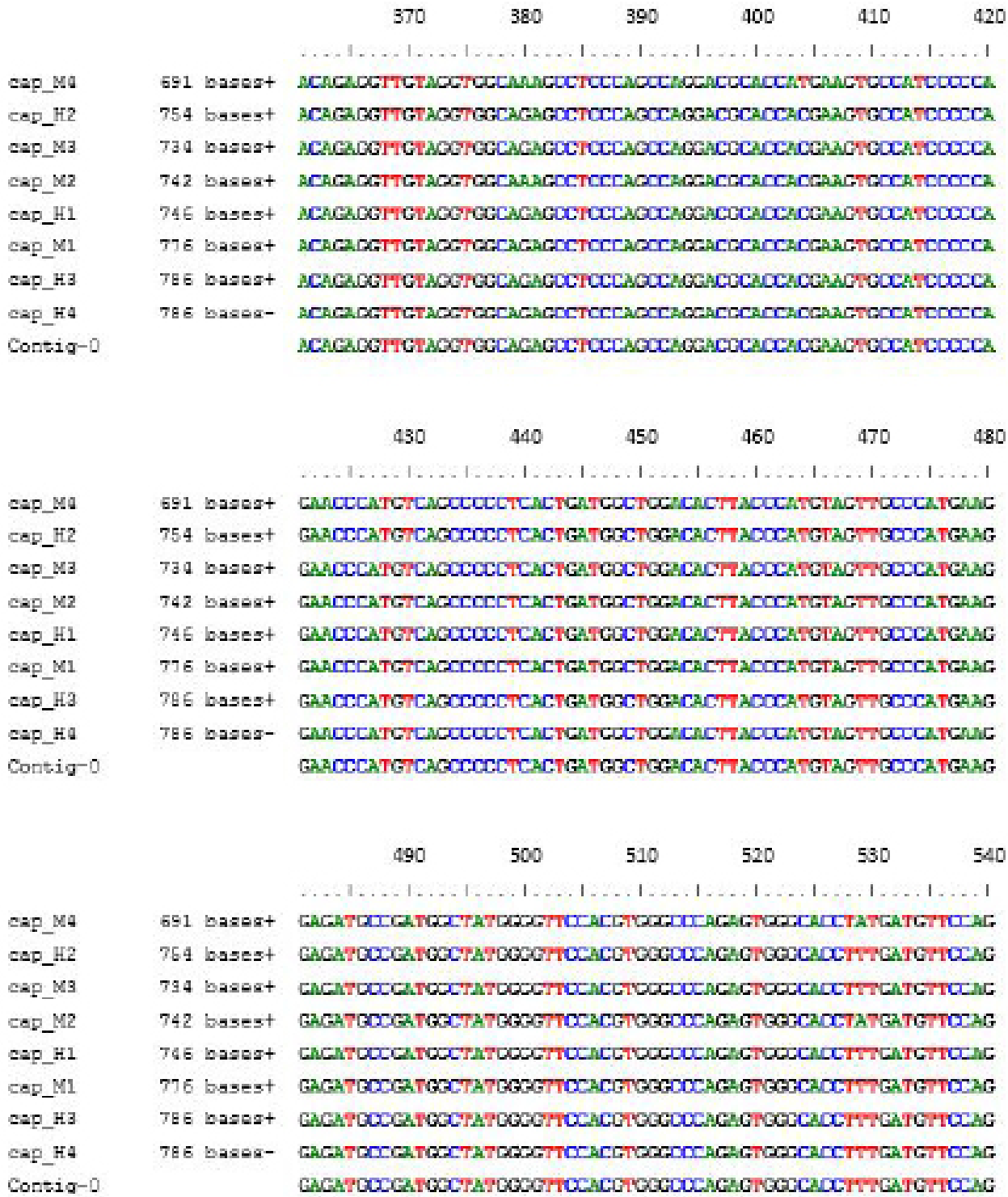
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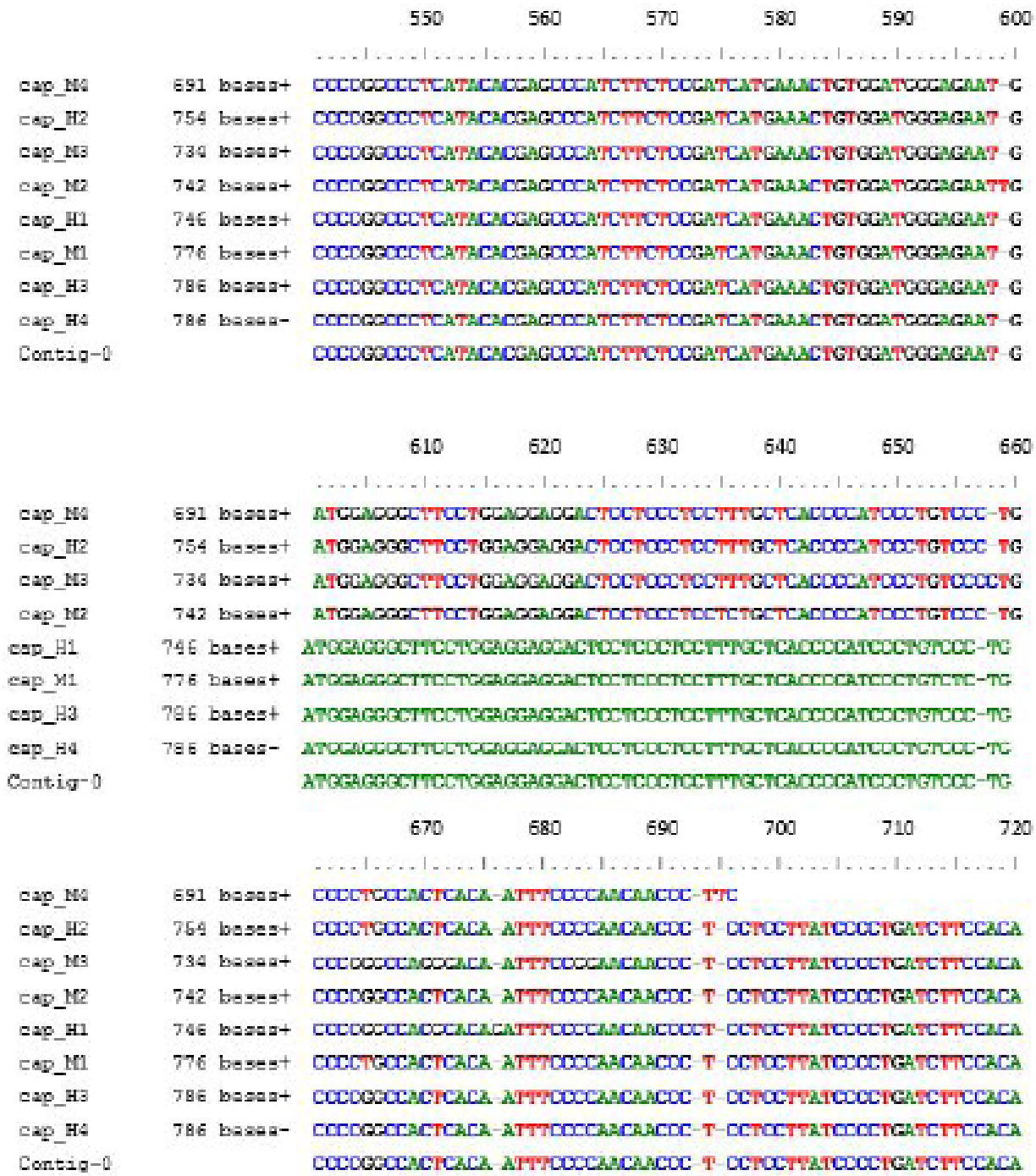

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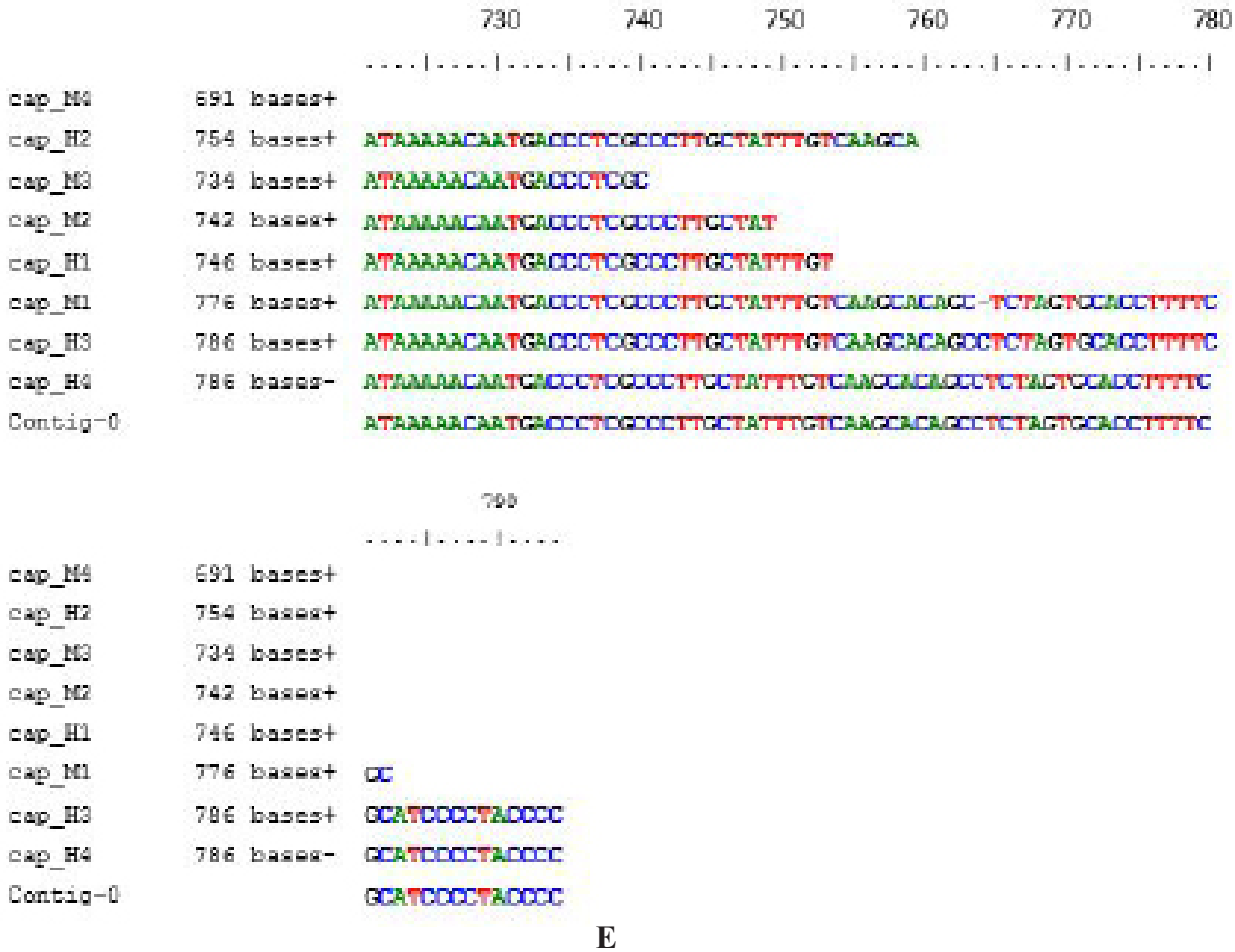

Figure 5. (A to E): Multiple Sequence Alignment of Partial PGLYRP-1 Gene Sequence of *Bubalus bubalis*. (Alignment of *Bubalus bubalis* PGLYRP-1 gene sequences with others, revealing variations at specific nucleotide positions between healthy and mastitis samples).

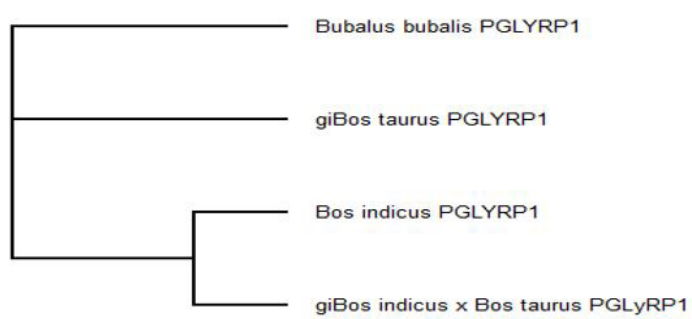

Figure 6. Phylogenetic analysis.

($P<0.05$) difference in somatic cell count of healthy and mastitis animals. So, further studies are needed with larger sample size, amplicon size and inclusion of more enzymes to get polymorphism in PGLYRP-1 gene.

The amplified PCR product of PGLYRP-1 gene was purified and successfully cloned into pJET1.2 blunt end cloning vector. The positive clones (recombinant plasmid having gene of interest) screened by touch colony PCR generated the expected 862 bp amplicon. The plasmid DNA isolated from the positive clones of touch PCR were sequenced in the automated DNA sequences. The generated sequence data was compared with the sequences available in NCBI database and BLASTn analysis revealed that the sequences of PGLYRP-1 gene showed 96% homology to that of *Bos taurus*, 96% of *Bos indicus*, and 96% to that of *Bos taurus* X *Bos indicus*.

Multiple sequence alignment of PGLYRP-1 gene sequence of *Bubalus bubalis* was carried out among other species to study sequence-based diversity globally. The alignment was carried out using ClustalW2 and Bioedit software. Randomly chosen six samples (three from healthy animal, three from mastitic animal) were sequenced using automated DNA sequences. The sequence comparisons between mastitis samples and samples from healthy animals reveal variations at specific nucleotide positions (i.e. G665T in M1, C158T, T246C, G380A, T529A in M2, C664G, T671G in M3, C158T, T246C, G380A, T529A, C404T, G665T in M4, T671G in H1, T58G, G665T in H2) as shown in Figure 5. The sequence data was generated and used for further analysis. In phylogenetic tree, partial sequence of PGLYRP-1 gene of *Bubalus bubalis* was found more closely related to *Bos taurus* then *Bos indicus* (Figure 6).

In Brief, the PCR amplification procedure for target region of PGLYRP-1 gene has been standardized which yielded consistent 862 bp products. Amplification of partial PGLYRP-1 gene using self design primers specific for cattle ortholog sequence, signifies that the locus is conserved in cattle and buffaloes. Partial sequencing of PGLYRP-1 gene of *Bubalus bubalis* (Murrah) has been done successfully. The PCR-RFLP revealed same pattern *viz*. xx, zz and yy of resolved digested fragments of PGLYRP-1 gene with all the restriction enzymes used i.e. HincII, TaqαI and ApaI respectively in both group of animals i.e. healthy and mastitis. As no polymorphism was observed, hence association of PGLYRP-1 gene polymorphism cannot be established with mastitis.

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