Screening the partial coding region of metallothionein isoform-2 gene in Zebu cattle

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Summary

Metallothionein (MT) is important because it binds tightly to heavy metals to decrease their toxicity. DNA was isolated from 30 toxic metal exposed and 30 toxic metal unexposed Zebu cows. The amplified metallothionein isoform-2 (MT-2) PCR product (489 bp) was further used for PCR-RFLP and DNA sequencing. MT-2 *Taq*I PCR-RFLP revealed homozygous genotype (AA) except for the E23 animal (AB). The genotype frequency of AA and AB (E23) genotypes in the exposed groups was 0.967 and 0.033 respectively. DNA sequencing was carried out for the toxic metal exposed sample (E23) and the control group sample (C13). Blast comparisons of the sequences were then aligned against a nucleotide database which revealed 150 nucleotide substitutions consisting of 70 transitions and around 80 transversions. DNA sequencing followed by PCR-RFLP for MT-2 revealed a higher number of nucleotide substitutions (150) for the AB genotype of E23 as compared to the AA genotype (38) of E21. The proportions of transversion mutations in the AB genotype were higher as compared to the MT-2 AA genotype. DNA sequencing was carried out based on random sampling for E21 and C13. Alignment analysis of the E21 and C13 sample revealed 38 nucleotide substitutions consisting of equal numbers of transition and transversion. BLAST analysis of the identified partial sequence revealed 89% identity with *Bos taurus*, 85% identity with sheep, 98% identity with buffalos and 100% identity with goat MT-2 sequences. Overall findings of the present study revealed DNA sequence variation in the coding region of the MT-2 gene of Zebu cattle which can be utilised to characterize and explore markers for heavy metal homeostasis in Zebu cattle.

Key words: DNA polymorphism, Metallothionein, PCR-RFLP, Sequencing, Zebu cattle

Introduction

Sustainable development of the ecosystem can be achieved by maintaining ecological balance. However, the fast pace of industrial development has disturbed the ecological balance as industrial pollution increases from the growing demand for electricity generation from industries and other sectors. Cattle population reared over generations in and around toxic metal emission sources is exposed to toxic metals and exhibits high incidences of stunted growth, patchy alopecia, anaemia, anoestrous and anorexia which lead to decreased productivity. In a study to investigate metal accumulation and detoxification processes in cattle from polluted and unpolluted areas, Roggeman *et al*. (2014) reported that cadmium and lead were significantly higher in tissues of cattle from polluted sites and cadmium seemed to be the most important metal that induced metallothionein (MT) in kidneys.

In this scenario, it becomes necessary to study genetic mechanisms for heavy metal homeostasis in animals reared around a toxic metal source. Several genes relate to heavy metal homeostasis in humans and animals; however, metallothionein is particularly important because it binds tightly to heavy metals to decrease their toxicity (Klaassen *et al*., 1999). The metallothionein gene is a low molecular weight (6-7 kDa), cysteine-rich, intracellular ubiquitous protein. There are four iso-forms of MT, namely MT-I, MT-II, MT-III and MT-IV; however, MT-I and -II are expressed in all tissues (Wu *et al*., 2007). The metallothionein gene has the highest affinity to essential metals zinc and copper as well as toxic metals such as cadmium and mercury (Robbins *et al*., 1991; Egli *et al*., 2006). The expression of the metallothionein gene starts by the binding of metal transcription factor-1 (MTF-1) to the regulative region of the MT gene known as metal responsive elements (MREs). The induction of the MT gene through the MREs region is initiated by several metal ions such as Zn, Cu and Cd (Sakulsak, 2012).

DNA-based polymorphism studies are constantly generating huge amounts of molecular genetic data, aiding in revolutionizing the genetic analyses of livestock species. Recently, part of the intronic region of the metallothionein gene was screened using PCR followed by sequencing in fly ash exposed and

unexposed cattle groups and revealed various single nucleotide substitutions (Doiphode, 2012). DNA polymorphisms in a number of environmentally responsive genes can explain variations in toxic metal biomarker values and health outcomes. Studies on mammals (wildlife, humans and rodents) show toxic metal exposures to be related to epigenetic marks such as DNA methylation (Basu *et al*., 2014).

Genetic variation leading to differences in expression and regulation of metallothionein proteins may contribute to observed differences among animals in terms of toxic metal uptake and metabolism. The metallothionein gene plays a significant role in heavy metal homeostasis; hence we decided to explore its coding regions because the expression of metallothionein starts by binding metal transcription factor-1 (MTF-1) to the regulative region of the metallothionein gene. A review of the literature revealed an absence of any report regarding polymorphism in the exonic region of the metallothionein gene in cattle exposed to heavy metals. Thus, to detect DNA polymorphisms, it seemed necessary to screen the coding regions of the MT gene which were not previously screened at the molecular level in cattle.

Materials and Methods

Experimental animals

The experimental animals were selected from two groups. The toxic metal exposed Zebu cattle group included 30 animals residing over generations and grazing in the close vicinity of a Thermal Power Station. The other unexposed group consisted of 30 Gaolao cattle, which resided far away from any toxic metal source over generations.

Blood collection and DNA isolation

Approximately, 8-10 ml blood from each experimental animal was collected under aseptic condition using sterile needles and centrifuge tubes containing 1.5 ml ACD (Acid Citrate Dextrose) anticoagulant (Himedia Lab., Pvt. Ltd., India) for half of the samples, and EDTA anticoagulant (Merck Specialties Pvt. Ltd., Germany) for the rest. The collected blood samples were stored at 4°C for further use for extracting DNA. The phenol, chloroform-isoamyl alcohol extraction protocol was performed (Clamp *et al*., 1993). The quality and quantity of isolated genomic DNA was assessed using agarose gel electrophoresis and a spectrophotometer, respectively. After quantification and quality check, all genomic DNA samples were stored as stock solutions at -20°C. A set of the working solution with a 50 ng/µL concentration for each genomic DNA sample was prepared to use as template DNA in PCR.

Primer designing

The primers were designed (Table 1) using PRIMER3 software (Untergrasser *et al*., 2012) for Zebu cattle metallothionein isoform-2 gene based on the *Bos taurus* database sequence available in NCBI. While designing PCR primers, care was taken to add a G-C rich sequence to the 5´ end of one of the primer pairs. All the designed primers were custom synthesized from a private company (Integrated DNA Technology, Germany). From these primers, the best working primer (MT-2) was used for PCR-RFLP and DNA sequence analysis.

PCR amplification

PCR amplification of the coding region of metallothionein isoform-2 was carried out by the optimization of the primer at the particular concentration of each PCR component. A total volume of 50 µL reaction mixture contains 10 pM/µL of each primer, 10 mM dNTP, 5 µL of 1 X PCR buffer, 1.5 units of Taq DNA polymerase (Sigma Aldrich, USA), 50 ng/µL of template DNA and Milli Q water to make up the reaction volume. A negative control without genomic DNA and a positive control of the known genomic DNA was always included in a set of reactions to confirm the accuracy of amplification. After centrifugation, PCR tubes were kept in the PCR thermal cycler (Applied Biosystems, USA) for amplification reaction by setting proper thermal cycling conditions. PCR thermal cycling conditions were: initial denaturation at 94°C for 10 min, cyclic denaturation at 94°C for 30 s, annealing at 56°C for 30 s, cyclic extension at 72°C for 45 s repeated for 40 cycles followed by a final extension at 72°C for 7 min. After completing the set PCR reaction programme, PCR tubes were taken out from the machine and electrophoresed in 2% agarose gel for 30 to 45 min and amplification was visualised in a GelDoc system (Bio-Rad, USA).

Selection of restriction digestion enzymes

The scanning for various restriction enzymes' cutting sites in reference sequences was carried out using RE Mapper software (http://www.restrictionmapper.org/). Restriction enzymes were selected based on the frequent cutting potential of restriction enzymes in the metallothionein isoform-2 reference sequence. Restriction enzyme *Taq*I (recognition site - TCGA) exhibited cutting sites at 58 bp, 244 bp, and 316 bp positions in the metallothionein isoform-2 reference sequence.

Restriction digestion and analysis

To detect DNA sequence variation in Zebu cattle

Table 1: Name, nucleotide sequence and PCR product size of designed primers

Name of primer	Nucleotide sequence	Product size (bp)
MT-2	FP: 5´CCAGCTCCTTTCCGCTATAA3´ RP: 5´GCACTTGCAATCTTTGCATT3´	489

FP: Forward primer, and RP: Reverse primer

using PCR-RFLP (Jiang and Gibson, 1999) in metallothionein isoform-II, 10 units of *Taq*I (New England Biolabs, UK) for metallothionein isoform-2 were added to 1 µg DNA PCR primer product and incubated at 65°C (*Taq*I) for 1 h. In total, 50 µL reaction mixtures, around 1.0 µg of PCR product, 5 µL of buffer and 44 µL of Milli Q water were added. Incubation was followed by heat inactivation at 80°C for 20 min.

The restriction digested PCR products were loaded for gel electrophoresis on 2.5% agarose gel along with a 100 bp DNA ladder, and electrophoresed for 30 and 45 min intervals at 80 V on a TAE buffer system. Gels were stained with ethidium bromide, the separated DNA bands were observed under UV trans-illuminator and images were analyzed under the GelDoc system (Bio-Rad, USA).

DNA sequencing and analysis

Selective PCR products were identified for custom Direct DNA sequencing (Eurofins Genomics India Pvt. Ltd.). Based on PCR-RFLP results, E23 (toxic metal exposed cattle group) and C13 (toxic metal unexposed cattle group) samples were selected for sequencing (Leonard *et al*., 1998). Similarly, E21 and C13 were selected randomly. The PCR product (100 ng) and primers (10 pM) were sent for sequencing. The obtained DNA sequences in FASTA format along with chromatographs were edited and analyzed using various bioinformatics tools including Bioedit (http://jwbrown.mbio.ncsu.edu/Bioedit/), BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), ClustalW omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) for the detection of nucleotide substitutions.

Results

DNA isolation and PCR

Good quality DNA was isolated by standard phenol, chloroform DNA extraction method. The nano drop readings at 260 A°/280 A° wavelength ratios for most of the samples were found to be in the range of 1.63 to 1.90 and the concentrations were in the range of 32-999.2 ng/µL.

The gradient PCR was set up at different annealing temperatures (ranging between 46-72°C) for the primer, for which the best amplification was noted at 56°C using 40 cycles. The amplified MT-2 PCR product (489 bp) representing the toxic metal exposed and unexposed group samples along with the negative controls were visualized for quality in 2% agarose gel electrophoresis (Fig. 1). These amplified MT-2 PCR products (489 bp) were further used for PCR-RFLP and DNA sequencing analysis.

PCR-RFLP analysis

The DNA concentrations of PCR products were measured and found to be between 40 to 1414 ng/µL. The final concentration of each PCR product was made uniformly as 1 µg/µL. Restriction enzyme *Taq*I (recognition site - TCGA) exhibited cutting sites at 58 bp, 244 bp, 316 bp positions in the metallothionein isoform-2 reference sequence in *Bos taurus*. On the basis of these 3 cutting sites, 4 bands were expected to be observed, i.e. 58 bp, 186 bp, 72 bp and 173 bp. However, in the present study, the cutting position in the *Bos indicus* MT-2 partial coding region was completely different compared to the reference gene sequence of *Bos taurus* cattle. The MT-2 gene PCR-RFLP using *Taq*I enzyme revealed a homozygous genotype (AA) with 180 bp, 86 bp and 65 bp size bands for all the animals except the E23 animal. This animal exhibited an AB genotype with an additional 270 bp band indicating polymorphism in the MT-2 partial coding region targeted (Fig. 2). The genotype frequency of the AB and AA genotypes was found to be 0.033 and 0.967, respectively, in the toxic metal exposed group. The frequency of the A allele was very high (0.9835) whereas the frequency of the B allele was found to be smaller (0.0165).

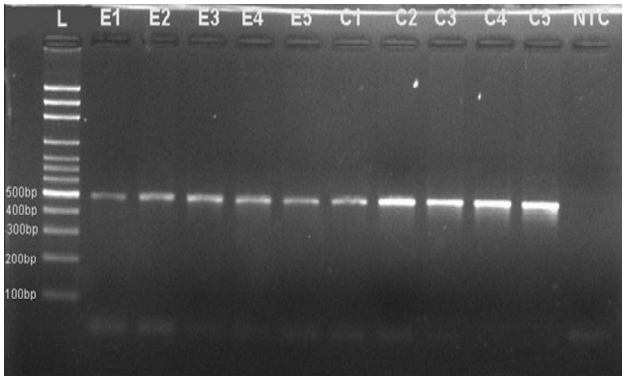

Fig. 1: PCR amplified product of metallothionein isoform-2 (size 489 bp) resolved in 2% agarose gel electrophoresis in Zebu cattle. L: 100 bp DNA ladder, E1-E5: Toxic metal exposed group cattle No. 1 to 5, C1-C5: Toxic metal unexposed group cattle No. 1 to 5, and NTC: Negative template control

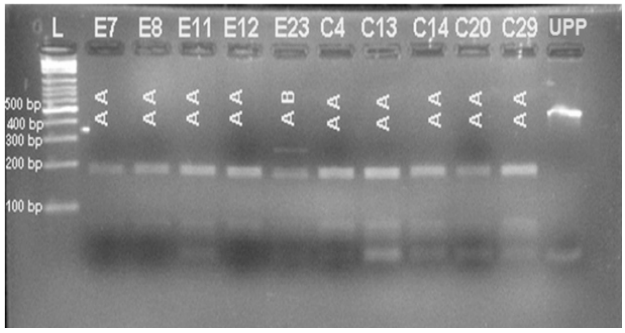

Fig. 2: Metallothionein isoform-2 polymorphism (E-23 exhibiting AB) using *Taq*I restriction enzyme digestion of 489 bp PCR product visualized in 2.5% agarose gel electrophoresis. L: 100 bp DNA Ladder, E7-E23: Exposed group cattle No. 7 to 23, C4-C29: Control group cattle No. 4 to 29, UPP: Undigested PCR product, and AA and AB: Homozygous and heterozygous genotypes

DNA sequence analysis

Selection of samples

Samples selected for sequencing based on PCR-RFLP results were E23 and C13. Similarly, E21 and C13 samples were selected randomly from both groups.

Selection Based on PCR-RFLP results

DNA sequencing results were obtained in FASTA and chromatograph format for the E23 sample representing the AB genotype from the toxic metal exposed group and C13 representing the AA genotype from the toxic metal unexposed group. CLUSTAL alignment analysis revealed 150 nucleotide substitutions among the E23 and C13 sample PCR product sequences. Out of these, 70 were transition mutations and 80 were transversion mutations. The chromatographic verification for each nucleotide substitution revealed 14 heterozygous and 9 homozygous mutations; however, some peaks were unanalysable.

Selection Based on random selection from both groups

E21 and C13 sample PCR product sequences were aligned using the CLUSTAL omega software which revealed 38 nucleotide substitutions. Out of these nucleotide substitutions, equal numbers of transition and transversion type mutations were observed (19 transition and 19 transversion). The chromatographic verification for each nucleotide substitution revealed 17 heterozygous and 12 homozygous mutations; however, some peaks were unanalysable.

Comparison of nucleotide substitutions among E23 and E21

The E23 sample representing the AB RFLP genotype exhibited 150 nucleotide substitutions, out of which 70 were transition and 80 were transversion. However, the E21 sample representing the AA RFLP genotype exhibited 38 nucleotide substitutions consisting of equal numbers of transitions and transversions. These DNA sequencing results followed by PCR-RFLP for metallothionein isoform-2 indicated a higher number of nucleotide substitutions (150) for the MT-2 AB genotype as compared to the AA genotype (38). The proportions of transversion mutations in the AB genotype were also higher compared to the MT-2 AA genotype. The comparison of single nucleotide substitutions found between the E-23 sample representing the MT-2 AB genotype and the E-21 sample representing the MT-2 AA genotype was carried out using sequence analytical tools. This revealed a higher number of nucleotide substitutions in the E-23 sample representing the AB genotype (150) consisting of a higher frequency of transversion type mutations (Table 2).

Comparison of Zebu cattle partial sequence with *Bos taurus* MT-2 reference sequence

The trimmed nucleotide sequence of metallothionein isoform-2 (420 bp) was aligned with NCBI reference sequence using the EMBOSS WATER pair-wise sequence alignment online tool which revealed 46.7% identity with the score value 445. The job EMBOSS WATER results for the pair-wise sequence alignment in context with the MT-2 partial coding sequence of Zebu and Hereford cattle were noted.

Phylogenic tree

The final aligned and edited nucleotide sequence of E21 was analyzed for homology search using BLAST revealing the nucleotide sequences of different species that were partially identical with the E21 nucleotide sequence. The BLAST analysis using 420 bp nucleotide sequence of MT-2 as the query sequence revealed 89% identity with the *Bos taurus* (e value 4e-136) MT gene, 85% identity with sheep (e value 2e-70), 98% buffalo (e value 3e-13) and 100% with goat (e value 3e-13). The relevant BLAST results were used to develop a phylogenic tree format which was edited using Bioedit software. The phylogenic tree based on the partial coding sequence revealed that the partial MT-2 Zebu cattle sequence was in closer proximity with sheep metallothionein sequence.

Discussion

It is necessary to study all industrial pollutants affecting the genetic health of animals grazing in and around pollutant sources. The present study intended to screen partial coding regions of the metallothionein gene in toxic metal exposed and unexposed Zebu cattle.

In the current investigation, DNA sequence analysis based on PCR-RFLP results and random sequencing from toxic metal exposed and unexposed groups revealed many single nucleotide substitutions. The DNA sequence analysis also revealed a higher frequency of transversion type mutations in the heterozygote PCR-RFLP genotype. The BLAST analysis of MT-2 as the query sequence revealed that it exhibited 89% identity with the *Bos taurus* MT gene, 85% identity with sheep, 98% with buffalo and 100% with goat. Wu *et al*. (2007) characterized yak metallothioneins nucleotide sequences to compare different species, and their results were found to be highly conserved. Wang *et al*. (2008) reported the MT-IV 189 bp coding sequence from rumen organs in both sheep and goats and submitted the results to Gene Bank. Yang *et al*. (2011) characterised the yak MT-III coding region in which BLAST results indicated that nucleotide sequences of yak share 98, 97, 96, 92, 91, 90, 89, 88 and 86% sequence similarities with cattle, milk goat, hair goat, pig, sheep, chimpanzee, human, dog and house mouse, respectively. Comparing homologies of MT-III sequences with MT-I and MT-II in yak, they found 69% and 67% homologies, respectively. This

Table 2: Comparison of single nucleotide substitutions between E-23 sample representing MT-2 AB genotype and E-21 sample representing MT-2 AA genotype

Sample No.	Total nucleotide substitutions	No. of transition	Frequency	No. of transversion	Frequency
E-23	150	70	0.466	80	0.533
E-21	38	19	0.5	19	0.5

conservation of motifs suggested a conservation of MT-III in molecular evolutions. Doiphode (2012) characterised partial intronic regions of metallothionein and reported a total of 96 nucleotide substitutions between Zebu cattle toxic metal exposed and unexposed groups. Fourteen individuals from the heavy metal exposed group indicated higher frequencies of the G-A nucleotide substitution, while the A-T nucleotide substitution was absent. Metallothionein intron-1 gene fragment exhibited a higher frequency of different nucleotide substitutions except for the T-A in cattle from the heavy metal exposed group.

In conclusion, the present study revealed mutations in the partial coding region of the metallothionein gene of Zebu cattle using PCR-RFLP and DNA sequencing molecular techniques. DNA sequence analysis using various bioinformatics tools revealed abundant nucleotide substitutions in MT-2 nucleotide sequences representing toxic metal exposed and unexposed animals. Due to the functional relevance of the metallothionein gene with toxic metal homeostasis, metallothionein is one of the important candidate genes to study in a context with toxic metal homeostasis. However, it seems necessary to analyze the relationship of single base pair substitutions in all regions of implicated genes with variable and precise phenotypic indicator traits like blood metal level etc. to identify genetic markers for heavy metal toxicity resistance and susceptibility mechanisms in larger sample sizes. Identification of genes for tolerance or susceptibility of toxic metals will be of immense importance for breeding and propagating superior genes. These findings may help explore metallothionein as one of the best DNA markers for toxic metal homeostasis in animals continuously exposed to toxic metals. In the future, identified polymorphisms within all genes related to heavy metal exposure along with relevant indicator/phenotypic traits/parameters can help explore molecular markers for toxic metal homeostasis.

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