Detection of Factor XI Deficiency (FXID) and Complex Vertebral Malformation (CVM) in Bali Cattle

S. W. Siswanti*ab, C. Sumantri*a, & Jakaria**

*a Department of Animal Production and Technology, Faculty of Animal Science, Bogor Agricultural University
Jln. Agatis, Kampus IPB Darmaga, Bogor 16680, Indonesia
*b Cipelang Livestock Embryo Centre, Directorate General of Livestock and Animal Health Services, Indonesian Ministry of Agriculture
Cijeruk, Cipelang 16004, Indonesia

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ABSTRACT

Factor XI Deficiency (FXID) is caused by imperfect insertion of poly adenine which is resulted in introduction of premature stop codon in FXI gene. Substitution of guanine into thymine in SLC35A3 gene caused Complex Vertebral Malformation (CVM). The research was aimed to detect the presence or absence of a genetic defect mainly CVM using SLC35A3 gene and FXID using FXI gene in Indonesian Bali cattle. The presence of this genetic defect may have a significant economic impact on the breeding program. The research of genetic defect was done mostly in dairy cattle, but there was no report for screening of genetic defect in Bali cattle. In this study, 303 fresh blood samples and 22 semen samples which were collected from Indonesian Bali cattle breeding center (BPTU HMT Denpasar, BPT HMT Serading West Nusa Tenggara and district Barru South Sulawesi) and artificial insemination centre (BBIB Singsosari and BIBD Baturiti) were used for screening of FXID and CVM. The amplicons of FXI gene were obtained by using PCR and that for SLC35A3 gene were obtained by using PCR-RFLP method with PstI restriction enzyme. These PCR products were analyzed by using 2% agarose gels electrophoresis. All genotypes were confirmed by DNA sequencing to determine an allele mutant. The allele mutant was not found in all of the samples. The result of this study showed that CVM and FXID were not detected in Bali cattle from Indonesian Bali cattle breeding and artificial insemination centres.

Key words: Bali cattle, genetic defect, SLC35S3 gene, factor XI gene

ABSTRAK

Insersi poli adenin yang tidak sempurna menyebabkan terjadinya kodon stop prematur pada gen FXI, sehingga terjadi kelainan genetik FXI deficiency (FXID). Substitusi guanin menjadi timin pada gen SLC35A3 menyebabkan kelainan genetik complex vertebral malformation (CVM). Penelitian ini bertujuan untuk mendeteksi ada atau tidaknya kelainan genetik terutama CVM menggunakan gen SLC35A3 dan FXID menggunakan gen FXI. Kelainan genetik ini biasa ditemukan pada sapi perah, tetapi sampai saat ini deteksi kelainan genetik FXID dan CVM belum pernah dilakukan pada sapi Bali. Sebanyak 303 sampel darah dan 22 sampel semen beku yang berasal dari pusat pembibitan sapi Bali (BPTU-HMT Denpasar, BPT HMT Serading West Nusa Tenggara dan district Barru South Sulawesi) dan pusat inseminasi buatan (BBIB Singsosari dan BIBD Baturiti) digunakan untuk mendeteksi FXID dan CVM. Amplifikasi gen FXI menggunakan PCR sedangkan gen SLC35A3 menggunakan PCR-RFLP dengan menggunakan enzim restriksi PstI. Produk PCR dianalisis dengan elektroforesis gel agarose 2%. Semua genotipe dikonfirmasi dengan DNA sekuensing untuk memastikan adanya alel mutan. Hasil penelitian tidak menemukan adanya alel mutan, baik dengan gen FXI, maupun SLC35A3. Hal ini menunjukkan bahwa tidak terdapat kejadian CVM dan FXID pada sapi Bali di pusat pembibitan sapi Bali dan balai inseminasi buatan.

Kata kunci: sapi Bali, kelainan genetik, gen SLC35S3, gen Factor XI

*Corresponding author:
E-mail: jakaria_karman@yahoo.co.id
INTRODUCTION

Bali cattle (Bos sondaicus, Bos javanicus, Bos/Bibos Banteng) is one of Indonesian native animal genetic resources which is domesticated descendent of the wild Banteng (Bibos banteng). Total population of Bali cattle are recorded 4,789,521 heads or 32.31% of all beef cattle in Indonesia (BPS 2011). Bali cattle are spreading throughout Indonesia, with the largest population found in the province of South Sulawesi, East Nusa Tenggara, West Nusa Tenggara and Bali with the percentage were 19.94%, 14.28%, 14.04%, and 13.31%, respectively.

An effort to make a superior Bali cattle was to maintain the existence and the uniqueness of Indonesian Bali cattle. The requirements for both superior dams and sires become very important thing in Bali cattle breeding programs. One of the requirements of bred cattle is free from genetic defect (Ministry of Agriculture, 2006). Genetic defect is undesirable in breeding program, due to the negative impact, such as decreased ability of production and reproduction, abnormal anatomy and some cases cause dying animal if the mutation is lethal (Meydan et al., 2010).

Improving the genetic quality of Bali cattle to produce dams and sires that were free from genetic defect are important to ensure that Bali cattle used for breeding are free of genetic abnormalities, mainly Factor XI Deficiency (FXID) and Complex Vertebral Malformation (CVM). FXID and CVM are an autosomal recessively inherited disorders that have been detected in dairy cattle (CVM). FXID in cattle was first discovered in Holstein cattle in Ohio (Kociba et al., 1969) and then spread to other countries because of the application of artificial insemination and semen trading. Deficiency of FXI in dairy cattle occurs because there is a mutation in FXI gene exon 12 in chromosome 27 with insertion of 76 nucleotide base (Ghanem et al., 2006; Marron et al., 2007). The other case of FXID in Japanese Black cattle occurred because there was an insertion of 15 nucleotide base in FXI gene exon 9 (Kunieda et al., 2005).

CVM is an inherited disorder during embryonic development causing abortion which affected in fetuses and perinatal death associated with vertebral abnormalities (Agerholm et al., 2004). CVM was first discovered in Danish Holstein Denmark in 1999 (Agerholm et al., 2001). Elite bull Carlin-M Ivanhoe Bell carried of this lethal mutation (Thomsen et al., 2006). The widespread of CVM in Holstein cattle was caused by the application of artificial insemination and semen trading. In breeding study, this malformation makes losses of pregnancies due to the large number of aborted foetus in 260 days of gestation that means large economical effect because of too many cows to be culled (Berglund et al., 2004). In the molecular base, CVM occurs because of substitution of guanin (G) base with timin (T) base at position 559 of cDNA of bovine solute carrier family 35 member 3 (SLC35A3) gene located at chromosome 3 (Thomsen et al., 2006).

Detection of genetic defects of CVM and FXID in Bali cattle in Indonesian breeding center has never been conducted in Indonesia, therefore this research needs to be done because Bali cattle is Indonesian native animal genetic resource which is an asset of the nation. The purpose of this study was to detect the presence or absence of a genetic disorder mainly CVM using SLC35A3 gene and FXID using FXI gene in Indonesian Bali cattle breeding center.

MATERIALS AND METHODS

Blood Samples and DNA Extraction

Blood samples (10 mL) were collected from each cattle by jugular vein puncture in tube containing EDTA. Genomic DNA was extracted using modified phenol-chloroform methods (Sambrook et al., 2001). DNA was stored at -20 °C until it used for PCR amplification (Table 1).

Pooling DNA

DNA pooling is a practical way in collecting and grouping many extraction DNA samples into one pool. Pooling allows allele frequencies in group of individuals to be measured using fewer PCR reaction and genotyping assays than in individual genotyping basis (Sham et al., 2002). Pooling DNA samples were done by combining DNA samples from several individuals for subsequent PCR analysis. One pool consist of five samples. To ensure that all samples were completely mixed, the pool of DNA were vortexed and centrifuged. If in one pool was obtained any mutant allele, then all members in one pool was checked one by one.

Polymerase Chain Reaction (PCR)

Amplification of polymerase chain reaction was performed using specific primers to the specific conditions of each gene. PCR assay for FXI gene was described by Marron et al. 2004 with minor modification. The sequences of forward primer are 5’ CCCACTGCGCTAGAATCGTT 3’ and reverse primer are: 5’ CAAGGCAATGTCATATCCAC 3’ (GenBank accession number: AH013749.2) (Figure 2).

Table 1. The sample origin, types and number of samples used in this study

| Sample origin       | Samples type | Sex Male | Sex Female | Total |
|---------------------|--------------|---------|------------|-------|
| BBIB Singosari      | Fresh Blood  | 28      | 0          | 28    |
| BPT-HMT Serading NTB | Fresh Blood | 41      | 11         | 52    |
| BPTU-HPT Denpasar   | Fresh Blood  | 98      | 59         | 157   |
| BBID Baturiti       | Frozen Semen | 22      | 0          | 22    |
| Barru District South Sulawesi | Fresh Blood | 48      | 18         | 66    |
| TOTAL               |              | 238     | 88         | 325   |
PCR assay for SLC35A3 gene was described by Kanae et al. (2005). The primer forward sequences for SLC35A3 gene are: 5’CACAATTTGTAGGTCTCACTGCA 3’, and primer reverse: 5’CGATGAAAAAGGAACCAAAAG GG 3’ (GenBank accession number: AY160683) (Figure 3). PIRA-PCR was used to detect a mutation that occurred in the CVM, which was conducted by inserting nucleotide bases on the primers used because of no restriction site in exon 4 BTA3 chromosome SLC35A3 gene (Kanae et al., 2005). This creation of the recognition site in primers has been introduced. The purpose of insertion of nucleotide bases was that new site could be new restriction site which could be indentified by restriction enzyme.

PCR were performed with a volume of 15 µL consisting of a pool of DNA samples in 1 µL, forward-reverse primer 0.3 µL (25 pmol each primers) (IDT Singapore), Go Taq® Green Master Mix 7.5 µL (PROMEGA Madison, WI U.S.A) and 6.2 µL of sterile water. The PCR was carried out using a thermocycler which was following this protocol; initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 20 s, annealing of primers at 60 °C for 20 s, and extension at 72 °C for 30 s and followed by final extension at 72 °C for 15 min. The amplification products were separated in 1.5% (w/v) agarose gels. Genotyping of FXID was resolved by electrophoresis of PCR products using 2% agarose gels which was followed by staining with ethidium bromide in TBE buffer for 40-45 min.

PCR-RFLP and Data Analysis

Detection of mutation in SLC35A3 gene exon 4 was performed by using the restriction enzyme PsI (Kanae et al. 2005). A total of 5 µL of PCR product was mixed with 0.7 µL of PsI buffer enzyme, 0.4 µL of PsI enzymes (5 U/µL) (Biolabs® Inc. New England) and 0.9 µL of sterile water. The mixture was incubated at 37 °C for 16 h.

To determine whether there was a mutant allele or not, electrophoresis on 2% agarose gels was conducted and followed by staining with ethidium bromide in TBE buffer for 40-45 min. The DNA bands produced from the process were then compared with marker to determine the length of fragments.

All genotypes were confirmed by DNA sequencing. Two samples were used for sequencing of both normal and carriers samples. Primer sequences used for analysis were primer forward and primer reverse. The sequencing results were analyzed by BioEdit program. The homology of FXI and SLC35A3 genes in GenBank were analyzed with nucleotide Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST). Determination of normal alleles and mutant alleles sequences of FXI gene and SLC35A3 gene were analyzed with Molecular Evolutionary Genetic Analysis (MEGA5) (Tamura et al., 2011).

Data were analyzed descriptively to determine the presence or absence of genetic abnormalities of CVM and FXID based on electrophoresis gels and sequences results from Bali cattle in Indonesian Bali cattle breeding centres.

RESULTS AND DISCUSSION

FXI Gene and SLC35A3 Gene Amplification

The primers used in this study were amplified DNA samples successfully. Amplicon size obtained was 245 base pairs (bp) for FXI gene and 287 base pairs (bp) for SLC35A3 gene (Figure 1). The primer position site used in FXI and SLC35A3 genes were showed in Figure 2 and Figure 3, respectively. Amplifications of SLC35A3 and FXI genes were successfully performed with annealing temperature of 60 °C with 35 cycles. PCR products of the SLC35A3 gene were subsequently digested with PsI restriction enzymes to detect the presence of mutations in these genes.

Detection of Mutation of Factor XI Deficiency (FXID)

In this study, a total of 325 Bali cattle was screened for FXI deficiency, all samples showed normal allele with

![Figure 1. DNA amplicon of Bali cattle in 1.5% agarose. Line 1-3= DNA band 245 bp for FXI gene; line 4-6= DNA band 287 bp for SLC35A3 gene; M= marker (100 bp).](image)

![Figure 2. Part of exon 12 on chromosome 27 within FXI gene of Bos taurus. The underline is primer position site, begin on 9200 to 9444 nucleotide. In mutant individual, there were 76 bases insertions began from 9402 composed of imperfect poly-adenine tract introduced stop codon. GenBank accession number: AH013749.2.](image)
length of 245 bp fragment (Figure 4). The normal FXI allele in unaffected animals produced 245 bp. Normal allele samples indicated that there was no affected animals with FXI deficiency (homozygous wild type). Analysis of 325 Bali cattle in breeding centre of Bali cattle in Indonesia revealed that all Bali cattle possessed normal genotypes (genotype FF).

In homozygous affected animals (genotype ff), the fragment had a length of 320 bp and the heterozygous (or carriers/genotype Ff) cattle exhibited two fragment of 244 bp and 320 bp due to the insertion of as many as 76 bp adenine rich fragment into exon 12 of FXI gene on chromosome 27. This insertion, composed of an imperfect poly-adenine tract followed duplicated region of the normal coding sequence, introduced a premature stop codon (TAA), which impaired the synthesis of functional protein (Marron et al., 2004). Patel et al. (2007) found that carrier individual exhibited two fragments with DNA length 247 bp and 324 bp due to the insertion of 77 base pairs insertion within exon 12 of the FXI gene. In this study, we did not find any cattle carrying this genetic defect.

Sequencing of the samples were also carried out to confirm whether these Bali cattle was carriers or not. BLAST analysis accessed from http://www.ncbi.nlm.nih.gov/BLAST was used to find the region of local similarity between sequences in GenBank. The BLAST search demonstrated 100% homology between FXI gene of
Bali cattle and Bos taurus, and obtained 245 bp whether Bali cattle as individual or as a pool of sample (Figure 5). The results confirmed that FXI gene of Bali cattle was identic with FXI gene of Bos taurus and pooling method was successfully detected mutation in the FXI gene in Bali cattle. Pooling methods were used for equipment and detection time efficiency (Yang et al., 2003) in a large number of samples (Sham et al., 2002). If one pool was analyzed and exhibited the different alleles, all members of the pool were analyzed one by one. This study obtained that DNA band in the individual sample showed the same results in a group of pooled samples.

The study of FXID in many breeds have been reported in many countries (Table 2). Monitoring of FXID in 103 heads of Polish Friesian-Holstein cattle with repeat breeding and mastitis showed that three of related cows were diagnosed as carriers of FXI gene. These carriers were identified as heterozygous cows and were healthy clinically (Gurgul et al., 2009). Investigation of FXID syndrom in 1001 heads of Indian dairy cattle showed that two holstein bulls detected as heterozygous for FXID, with carrier frequency of 0.6% (Patel et al., 2007). The carriers frequency of the FXID also detected in Turkey, USA, Republic Czech, Iran, Japan with carrier frequency of 1.80%, 1.20%, 0.36%, 2.91% and 2.50% repectively (Meydan et al., 2010; Marron et al., 2004; Cítek et al., 2008; Gurgul et al., 2009; Ghanem et al., 2005).

There was a little frequency of FXID carriers in many countries among cattle breeds.

| Breed Description          | Number of samples | Carriers (Number, %) | References          |
|----------------------------|-------------------|----------------------|---------------------|
| Bos taurus                 |                   |                      |                     |
| Friesian Holstein          |                   |                      |                     |
| Turkey                     | 225               | 4 (1.80)             | Meydan et al. 2009  |
| Turkey                     | 350               | 4 (1.20)             | Meydan et al. 2010  |
| USA                        | 419               | 5 (1.20)             | Marron et al. 2004  |
| India                      | 330               | 2 (0.60)             | Patel et al. 2007   |
| Japan                      | 40                | 1 (2.50)             | Ghanem et al. 2005  |
| Republic Czech             | 279               | 1 (0.36)             | Cítek et al. 2008   |
| Poland                     | 103               | 3 (2.91)             | Gurgul et al. 2009  |
| Iran                       | 100               | 0 (0.00)             | Eydivandi et al. 2011|
| Cross breed                |                   |                      |                     |
| FH × B. Indicus            |                   |                      |                     |
| India                      | 265               | 0 (0.00)             | Patel et al. 2007   |
| Jersey × B. Indicus        |                   |                      | Patel et al. 2007   |
| Bubalus bubalis            |                   |                      |                     |
| Iran                       | 300               | 0 (0.00)             | Bagheri et al. 2012 |
| India                      | 153               | 0 (0.00)             | Patel et al. 2007   |
| Japanese Black Cattle      |                   |                      |                     |
| Jepang                     | 123               | 51 (47.15)           | Ohba et al. 2008    |
| Bos indicus                |                   |                      |                     |
| India                      | 79                | 0 (0.00)             | Patel et al. 2007   |
| Khuzestan native cattle    |                   |                      |                     |
| Khuzestan                  | 230               | 0 (0.00)             | Eydivandi et al. 2011|
| Bali cattle                |                   |                      |                     |
| Indonesia                  | 325               | 0 (0.00)             | Present study       |

FXID deficiency was not detected in Iranian buffalo (Bagheri et al., 2012), Bos indicus, Crossbred of Friesian Holstein and Bos indicus, Jersey, Crossbred of Jersey and Bos indicus, Bubalus buBalis (Patel et al., 2007), Khuzestan native cow and Iranian Holstein cattle (Eydivandi et al., 2011). Similar finding in Bali cattle in Indonesia that there was no detected carriers of FXID in Indonesian Bali cattle breeding centre in this study. FXI Deficiency was also found in Japanese Black cattle caused by the insertion of 15 nucleotides within exon 9 of the gene to FXI (Kunieda et al., 2005), so the case of FXID between Holstein cows and Japanese Black Cattle is a different case, which might be caused by the differences in mutation of FXI gene, characteristic of different types and breeding environment (Ohba, 2008).

FXID in cows have a tendency to show repeat breeding (Ghanem et al., 2005), abberation in reproductive performance, such as lower calving rate and lower calf survival rate, small diameter of follicular accompanied by lower peak estradiol concentrations in plasma near the time of ovulation (Liptrap et al., 1995). Japanese black calf with FXI deficiency was indicated by hemostatic deficiency, growth retardation and hip dysplasia. FXID in Japanese black cattle might not show any symptoms by itself, but it could be shown by hemorrhage symptoms in association with other factors (Ohba et al., 2008).

The results from this study revealed that all of Bali cattle reared in Indonesian Bali cattle centres
Mutation Detection of Complex Vertebral Malformation

In this study, a total of 325 samples of Bali cattle were screened for CVM. The primer used in this study successfully amplified DNA fragments of 287 bp. The PCR products of CVM were digested with \textit{PstI} restriction enzymes. Restriction enzymes \textit{PstI} that were used in this study were able to digest nucleotide sequences at sites 5’CTGCA↓G 3’. Electrophoresis in 2% agarose gels of all of the PCR products exhibited one fragment of 264 bp (Figure 6, fragment 23 bp could not be seen). Analysis of all of PCR products could be digested by \textit{PstI} restriction enzymes. These results indicated that all of the samples were unaffected animals, (homozygous normal animal: GG). If the PCR product could not be digested by \textit{PstI} restriction enzymes, it indicated that the individual sample contained the mutant alleles (affected individual) (Kanae et al., 2005). In affected animals, it will be found one fragment of DNA with the length of 287 bp (homozygous mutant animal: TT). In carriers animal it will be obtained three fragments of 287 bp, 264 bp, and 23 bp (heterozygous animal: GT). It showed in this study that all of the Bali cattle was screened with SLC35A3 gene possessed the normal genotypes. Bali cattle reared in Indonesian Bali cattle breeding centres produced the superior Bali cattle whether male or female, all the samples were normal individual with undetected CVM genetic defect.

Sequencing of the samples were also done in this study in order to confirm whether these cattle were carriers or not. BLAST analysis used to find the region of local similarity between sequences. The BLAST revealed 100% homology with the XI SLC35A3 gene of \textit{Bos taurus} and obtained 287 bp whether Bali cattle as individual or as a pool of sample (Figure 7). Alignment of nucleotide sequences of Bali cattle with \textit{Bos taurus} suggests that CT is intentionally inserted mismatch nucleotides in the forward primer, it means that the primer modified with artificial restriction sites succesed for being new restriction site that can be recognized by restriction enzymes used.

CVM commonly found in Holstein cattle, however, it’s possible to occur also in cattle from other breeds, both male and female, adult and calve (EPS, 2006). The frequency of CVM carriers have been reported in many countries (Table 3). CVM detection in Chezch showed that in 111 Chezch elite holstein cows, 21 were heterozigous for CVM (18.9%) (Čítek et al., 2006), carrier frequency of CVM in Polish, Denmark, Japan, Slovak, Swedish, Turkey and Chinese were 24.79%, 31.00%, 31.00%,
13.00%, 8.50%, 23.00%, 3.40% and 43.58% respectively (Rušč et al. 2007; Thomsen et al. 2006; Ghanem et al. 2008; Gábor et al. 2008; Berglund et al. 2004; Meydan et al. 2010; Chu et al. 2008). The CVM carriers in dairy cattle were high frequency (20%-30%). This result is an alarm that this genetic defect was widespread in the world.

PIRA-PCR method that was used in this study is one of the methods for detecting CVM that used to perform inserting an artificial restriction sites on the primers used to replace nucleotide bases in the sequences of primers used in order to be recognized by the restriction enzyme used. This technique could be used to detect single base mutation in a gene that does not have restriction sites around the mutated region. CVM was the genetic defect occurred due to point mutations cause a point mutation (missense mutation) that is due to the substitution of guanine into thymine (Kanae et al., 2005) resulting in codon changes that lead to changes in the amino acid valine (GTT) into phenylalanine (TTT) (Thomsen et al., 2006). Base sequences from GenBank accession number AY160683, nucleotide base position in 9871 was a point of mutation, in normal animals it was guanin base (G) and in affected or carrier animals guanine base (G) change to thymine (T). Figure 7 showed that there was no change of guanine (G) to thymine (T) in SLC35A3 gene within exon 4 of Bali cattle in this study. This result indicated that the samples which were taken from Indonesian Bali cattle breeding centres were free from CVM.

The Implementation of Screening Genetic Defect CVM and FXID in Bali Cattle in Indonesian Bali Cattle Breeding Center

Although there was no mutant allele detected for FXI deficiency (FXID) and Complex Vertebral Malformation (CVM) in Bali cattle at Indonesian Bali cattle breeding centres, it is important that screening of the genetic defect in breeding centres is conducted continuously. This is because those places are the centres of Bali cattle, both female and male that are used for dams and sires to produce the superior Bali cattle. The screening test of genetic defect especially for sires is very important to make sure that Bali cattle used to produce semen for artificial insemination program are free from genetic defect that might be found in the progeny.

Other important things that should be done to optimize the breeding program is production and reproduction record of the animals. It is important to record every case of production and reproduction to ensure that the cattle is superior. The screening of genetict defect also needs the necropsy record from any dead animals.

Besides CVM and FXID, there are several inherited bovine disorders that should be screened in breeding systems such as Bovine Leukocyte Adhesion Deficiency (BLAD), Bovine Citrulinemia (BC) and Deficiency of Uridine Monophosphate Synthase (DUMPS) (Meydan et al., 2010).

CONCLUSION

There were no carriers of FXID (Factor XI Deficiency) and Complex Vertebral Malformation (CVM) in Bali cattle at Indonesian Bali cattle breeding centres (BPTU-HMT Denpasar, BPT-HPT Serading West Nusa Tenggara, VBC district Barru South Sulawesi) and artificial insemination centre (Balai Besar Inseminasi Buatan Singosari and BIBD Baturiti).

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