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RESEARCH ARTICLE

Correlation between Osteopontin Promoter Gene and Fresh Semen Quality in Friesian Holstein Dairy Cows

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ABSTRACT:

This study aims to investigate the polymorphism of osteopontin promoter gene in Holstein Friesian (FH) dairy cows and its correlation with quality of fresh semen in FH dairy cows. The basis determining for osteopontin as the main bio-marker in the determination of male Holstein dairy cow fertility by several previous studies which showed that osteopontin levels in seminal plasma Holstein dairy cows with a good fertility have osteopontin concentrations higher than the low fertility of dairy cows. A total of 14 blood samples taken from Holstein Friesian dairy cows then the DNA extracted and amplified using primers SPP1F and SPP1R. The 306 bp band as a target was detected in all 14 samples then sequenced for analysis of the nucleotide base. The results showed that all samples with low sperm concentration occurred deletion at10080 bp and the transition (G-A) at 10090 bp. The results indicated that this mutation site could be related with the trait susceptibility to sperm concentration. Further studies are needed to address other parameter related to comprehensive sperm quality.

KEYWORDS: Polymorphism, Osteopontin, Male fertility, dairy cows.

INTRODUCTION:

Examination of semen in males as a measure of fertility rates have only done through macroscopic and microscopic examination. In addition to such examinations can also be seen from the pedigree, namely the selection of which is based on the reputation demonstrated by the ancestors of cows is concerned but the test is less accurate, because a bloodline or the offspring of individuals who either does not necessarily mean that the characteristics of good will selection and inherited through marriage. Another male fertility test that is based on progeny test is a test to see heritability trait but this test takes approximately 4-6 years old so it is not efficient[1,2,3].

Basic determination of osteopontin as a bio-marker principal in determining the fertility of dairy cows Holstein bull is based on some earlier research which showed that the levels of osteopontin in seminal plasma Holstein dairy cows with a good fertility has a concentration of osteopontin 2.5-fold when compared with the low fertility of dairy cows. Erikson *et al.*, (2007) said that the males are inspected dairy cows andfresh cement containing osteopontin has a great potential in the success of fertilization in vitro and in vivo than not containing osteopontin. This is reinforced by previous studies that prove the relationship between osteopontin with fresh semen quality dairy cows FH in Indonesia[4,5].

Osteopontin gene promoter polymorphism associated with male fertility in dairy cows Holstein. Some evidence suggests that there is a relationship between gene polymorphisms promoter osteopontin, one of which is polymorphism promoter osteopontin motility and viability of spermatozoa fresh semen cow. Osteopontin gene promoter region determination based on previous research conducted by Rorie *et al.*, (2016) identified seven SNPs region that acts as a promoter osteopontin, among others: 3379 bp, 3490 bp, 3492 bp, 5075 bp, 5205 bp, 5209 bp and 5263 bp of osteopontin gene promoter. Then, substitution thymine to guanine at 3379 bp correlated with an increase in the percentage motility

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spermazoa, but for the viability parameters have not been identified. Morales *et al*, (2013) in a research report that testing of males routinely in the IB program is very important as an indicator of fertility[6,7].

Development of dairy cow population ahead should be selected based on the value of breeding (breeding value), the use identifier genetically especially those controlling reproduction and application of BSE so stud dairy cattle FH in the Central IB can be accounted for its quality, because the male who will spread the trait superior to the population. Development testing of the study through the identification identifier genetically to semen quality has been done in some developed countries, it is expected to support the accelerated population of dairy cows FH superior in quality. Genetic selection system requires the identification of the genetic identifiers as candidate genes that control reproductive traits, especially the bulls[8,9].

MATERIAL AND METHODS:

Tools and materials:

The tools used in the study include a glove, mask, ice box, paper labels, microsentrifuge tube (1.5 mL), micro PCR tube (200 mL), micropipette, white tip, yellow tip, vortex engine, sentrifugator, incubator CO2, freezer, thermocycler, EDTA, Horizaontal SDS-PAGE (Biorad), Gel Documentation (Biorad), thermocycler (Biorad), Nano-200 Micro-spectrophotometer nucleic acid. Materials used in research FH male cattle blood samples, Genomic DNA mini kit tissue, ddH2O, forward primer (SPP1_F) 5'-GCAAATCAGAAGTGTGATAGA-3 'and reverse primer (SPP1_R) 5'-CCAAGCCAAACGTATGAGTT-3', the PCR mix, DNA ladder 100 bp and 1 kb, TBE, agarose 1% and 2%, loading dye, alcohol 70%, aluminum foil, red gel.

Cow Blood Sample Selection FH Males:

Samples taken in the form of blood samples of 14 Holstein Friesian dairy cows 3-5 years old adult male obtained from BBIB Singosari, Malang, East Java, Indonesia, and local cattle ranchers.

Table 1: Males FH Cow Sample Data

No.	Origin	Code
1	BBIB	2
2	breeder	7
3	breeder	8
4	BBIB	17
5	breeder	19
6	breeder	D
7	breeder	H
8	BBIB	I
9	BBIB	J
10	BBIB	L
11	BBIB	M
12	BBIB	N
13	BBIB	13
14	BBIB	14

Cow Blood Sampling FH Males:

Location blood sampling performed on coccygea vein. The volume of blood samples are taken as 3cc of each individual bull. The blood sample is inserted into EDTA vacutainer tube and labeled according to the name of the individual samples of cattle. Samples were then stored at a temperature of 4^0C. Storage of whole blood samples at a temperature of 40°C have been selected for the distance between sampling and DNA isolation process is not too long.

After blood collection examination fresh semen quality with cement collected in advance. Fresh semen collection is done by using an artificial vagina. Artificial vagina is prepared with an internal temperature of 45ºC and already smeared with vaseline. The males are lured by using dummy / female pemancik then proceeds semen collection examined macroscopically immediately include: volume, color, odor, pH and viscosity and microscopic examination.

Isolation of DNA:

Isolation of DNA from blood samples of male cattle FH using insulation kit from Geneaid namely Genomic DNA mini kit tissue and blood. The main principle in the isolation of DNA, there are three namely the destruction (lysis), DNA extraction or separation of solid materials such as cellulose and proteins, and DNA purification [10,11].

Quantity and Quality Test DNA:

DNA quantity insulation test results done using machine Micro-Nano-200 spectrophotometer nucleic acids. Shells TE buffer used is obtained from a kit geneaid, TE buffer is dripped directly onto the pedestal submicroliter cell as 1µL, then absorbance is measured by pressing a button blank blank after the lid (cover) is closed. The wavelengths used are 260 nm and 280 nm. An initial stages, a total of 1 mL sample was dropped on pedestal submicroliter cell that has been cleaned using a tissue. Lid closed above the sample was dropped and pressed the button sample and then wait until the results come out on the screen. To sample the stages are the same as above, done up to the last sample. Data out in the form of numbers or graphs.

Primer design:

Primers used for DNA amplification by polymerase chain reaction technique (PCR) was designed using NCBI Genebank: AY878328.1. Forward primer and reverse primer obtained through primer 3plus using data AY878328.1 with 12,300bp linear DNA. A pair of forward primer (SPP1_F) 5'-GCAAATCAGAAGTGTGATAGA-3 '(Length: 21 bp, Tm: 53.7, GC: 38.1% and the reverse primer (SPP1_R) 5'-CCAAGCCAAACGTATGAGTT-3' (Length: 20 bp, tm: 56.3, GC: 45%).

The process of Polymerase Chain Reaction (PCR):
DNA samples of steers was amplified using the PCR method. A pair of primers used forward primer (SPP1_F) and reverse (SPP1_R). PCR amplification dengna machine (Biorad) begins by mixing the DNA 1µL, 1µL 10 pmol forward primer, 10 pmol reverse primer 1µL, 5µL PCR mix and 2 mL ddH_2O into microtube 200 mL. According Zuhriana (2010), amplification stages starting from predenaturation 94^0C for two minutes, denaturation 94^0C for 30 seconds, and then annealed at a temperature of 55-60^0C for 30 seconds. Extension at a temperature of 72^0C for 30 seconds and post extension at 72^0C for 7 minutes. The process will be repeated for 30-35 cycles[12].

Purification of PCR Products:
Purification of the PCR product aimed to purify DNA and eliminate the remnants of PCR mix covering dNTPs, Taq polymerase, Mg ions, as well as ddH_2O and PCR primers located within the tube. The method used in the purification protocol was modified Santella by ethanol precipitation.

DNA sequencing:
Sequencing of the PCR product of the gene Osteopontin be two-way, namely by using a primer SPP1_F 10 pmol and 10 pmol SPP1_R to see osteopontin gene sequences were amplified using dye terminator method. PCR product DNA concentration of at least 50 ng / mL to do sequencing. Sequencing the form of a graph representing the content of adenine, thymine, guanine and cytosine contained in the DNA fragment that had been labeled by ddNTPs.

Data analysis:
Analysis of the data used is NCBI Blast, Bioedit and MEGA 7.0. Through the NCBI Blast program can know the percentage of homology and molecular variation in isolates a sample of SNPs (Single Nucleotide Polymorphism) such as insertions, deletions, and substitutions (transition or transversion) by aligning the results of the fourth sample sequence with the NCBI database Genebank: AY878328.1 alignment using algorithm Clustal W multiple allignment. Further analysis of the molecular variation isolates the sample is to use Bioedit program to see what kind of mutation that occurs, the type of amino acid and nucleotide positions that have mutations in the sample isolates. Phyilogenic analysis using MEGA version 7.0 software with bootstrapped Neighbor-Joining method (NJ) uses 1000 times repetition. The results of the analysis of the MEGA program will be acquired genetic distance matrix equation of bases.

RESULTS:

Semen Quality Inspection FH Fresh Dairy Cattle:
Fresh semen examination was conducted on the examination of the volume, color, odor, pH and concentration. Fourteen fresh semen of male dairy cows examined three times. Data quality fresh cement were shown in Table 2.

Table 2: Semen Quality Inspection of Males FH

Code	The average of fresh semen volume (ml)	Color and Odor	pH	The mean concentration of fresh cement / ml	Overall quality
2	6.9	Normal	6.8	1.031,4x106	Good
7	5.3	Normal	6.8	799.7 x106	Good
8	10.1	Normal	6.8	705.7 x106	Good
13	6.2	Normal	6.8	1418.7 x106	Good
14	11	Normal	6.8	1090 x106	Good
17	5.4	Normal	6.8	1075 x106	Good
19	3.8	Normal	6.8	429.4 x106	Poor
D	5.1	Normal	6.8	755.3 x106	Good
H	4.1	Normal	6.8	286.6 x106	Poor
I	5.8	Normal	6.8	1345.9 x106	Good
J	5.9	Normal	6.8	1173.9 x106	Good
L	7.3	Normal	6.8	1290 x106	Good
M	6.2	Normal	6.8	1142.7 x106	Good
N	4	Normal	6.8	312.4 x106	Poor

Isolation of DNA from blood samples FH Cow Males:
Isolation of DNA is done using a blood sample by using Genomic DNA mini kit in accordance with the procedure tissue. Results obtained in the form of total DNA extraction which further test the quantity and quality. Test quantity by machine Micro-Nano-200 Nucleic acid spectrophotometer with a wavelength of 260 nm and 280 nm. DNA total yield of isolation is then used for the amplification process osteopontin from bulls with the PCR technique to determine the sequence of the gene osteopontin from some bulls, so as to know their gene polymorphism promoter osteopontin in dairy cows Holstein Friesian (HF) and its relationship to the level of fertility spermatozoa, as indicated by the quality of dairy cows FH spermatozoa from fresh cement.

Table 3: The Concentration and Purity of DNA Total Cattle FH Males

Sample Code	Concentration ng / mL	Purity
2	308	1.92
7	190	1.91
8	223	1.81
13	254	1.87
14	302	1.89
17	201	1.91
19	210	1.81
D	254	1.87
H	338	1.98
I	176	1.91
J	220	1.81
L	271	1.88
M	289	1.80
N	198	1.77

Osteopontin Gene amplification by PCR Method:
Osteopontin gene amplification is done to increase the osteopontin gene fragment prior to sequencing so that it can be used to determine the osteopontin gene sequences FH male cattle. Primers used to amplify the gene osteopontin taken from Genebank with number sequences AY878328.1 and designed using primer3plus program, so we get a forward and reverse primer pair shown in Table 4. PCR method used in this study includes the step pre denaturation, denaturation, annealing, extension and post-extension with suu and time listed in Table 5.

Table 4: Primary Nucleotide Sequence Cow Osteopontin gene FH Males

Primary	Oligo Nucleotide Sequence
forward (SPP1_F)	GCAAATCAGAAGTGTGATAGA 5'-3 '
Reverse(SPP1_R)	CCAAGCCAAACGTATGAGTT 5'-3 '

Table 5: PCR program for cattle Osteopontin Gene Amplification FH Males (35x cycle)

Condition	Time	Temperature
Pre denaturation	4 minutes	94 ºC
Denaturation	30 seconds	94 ºC
Annealing	30 seconds	55-69 ºC
Extensions	30 seconds	72 ºC
Post extension	7 minutes	72 ºC

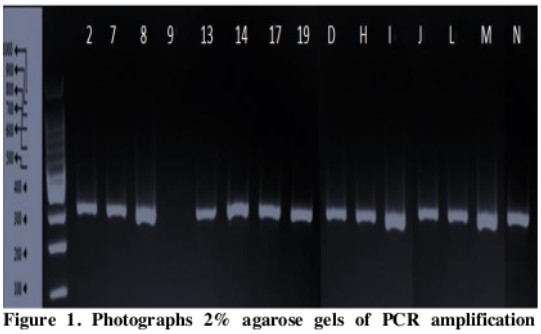

Figure 1. Photographs 2% agarose gels of PCR amplification products with a target of 306bp band was detected in all samples (n = 14) (Gel Doc, Biorad).

The results of the purified PCR products subsequently aim to purify DNA and eliminate the remnants of PCR mix, and ddH_2O primer inside the PCR tube. The method used in purification is ethanol precipitation with Santella protocol modification. The results of the purification product then sequenced the DNA of the gene osteopontin be two-way, namely by using a primer SPP1_F and SPP1_R to see osteopontin gene sequences were amplified.

Osteopontin Gene Sequence Analysis Results:
Sequencing the form of graphs that show the content of adenine, thymine, guanine and cytosine contained in the DNA fragment and format data in the form fasta. The fourth sample is inserted into the NCBI BLAST program for alignment of the sequencing results with NCBI Genebank AY878328.1. The alignment results of that magnitude ident and alignment on the sample base with NCBI database Table 6.

Table 6: Results of alignment with NCBI database

Sample Code	Identity (%)
2	99
7	99
8	99
13	99
14	99
17	99
19	99
D	97
H	98
I	99
J	99
L	99
M	99
N	98

Results of identity of all samples above 95%, it shows that all the samples had good similarity with the NCBI AY878328.1.Osteopontin gene sequences of all male cattle FH aligned with the reference gene osteopontin AY878328.1 NCBI database. Sample sequences aligned sequence starting from the base to 9900-10092.

```
                    9910       9920       9930       9940       9950
AY878328.1  AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
D_SP1R      AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
H_SP1R      AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
I_SP1R      AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
J_SP1R      AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
L_SP1R      AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
M_SP1F      AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
N_SP1R      AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
2_SP1R      AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
7_SP1R      ---------- ---------- ---------- ---------- ----------
8_SP1R      AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
13_SP1R     AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
14_SP1R     AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
17_SP1R     AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
18_SP1R     AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT
19_SP1R     AGAGAGTCAC CTTTTGATTA TCCAGGCTAA TAGGGAGGTG ATTTTAGTTT

                    9960       9970       9980       9990      10000
AY878328.1  TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
D_SP1R      TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
H_SP1R      TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
I_SP1R      TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
J_SP1R      TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
L_SP1R      TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
M_SP1F      TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
N_SP1R      TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
2_SP1R      TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
7_SP1R      ---------- ---------- ---------- ---------- ----------
8_SP1R      TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
13_SP1R     TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
14_SP1R     TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
17_SP1R     TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
18_SP1R     TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT
19_SP1R     TGGGGGTGTG CATTAATACA TGGATTCTCT GATCCCCTGA GAATTTTCAT

                   10010      10020      10030      10040      10050
AY878328.1  TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTATTGGATC
D_SP1R      TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTACTGGATC
H_SP1R      TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTACTGGATC
I_SP1R      TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTACTGGATC
J_SP1R      TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTATTGGATC
L_SP1R      TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTACTGGATC
M_SP1F      TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTACTGGATC
N_SP1R      TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTACTGGATC
2_SP1R      TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTACTGGATC
7_SP1R      ---------- ---------- ---------- ---------- ----------
8_SP1R      TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTATTGGATC
13_SP1R     TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTATTGGATC
14_SP1R     TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTATTGGATC
17_SP1R     TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTACTGGATC
18_SP1R     TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTACTGGATC
19_SP1R     TTCAAATAGA AAAGGTAGTC TCACAATTAT GTATCTGTAT TTATTGGATC
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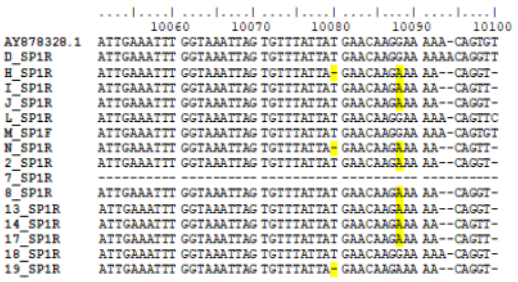

Figure 2. The result of the alignment of the nucleotide bases using Bioedit program

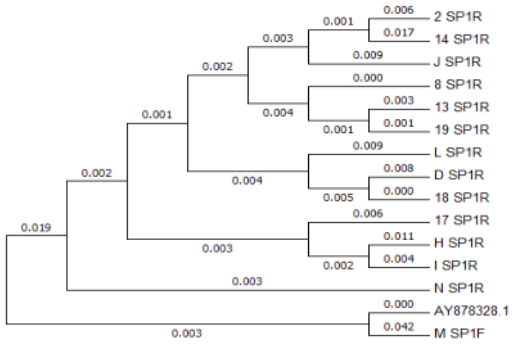

Figure 3. Cattle phylogenetic tree male FH Neighbor-Joining method with Bootstrap replication 1000x.

Based on the phylogenetic tree in Figure 3, there are known two major clade. The first clade consisting of the sample with the code 2, 14, J, 8, 13,19, L, D, 18, 17, H, I, and N. As a second clade consisting of NCBI AY878328.1 and sample M. All sample shows the results of genetic distance with NCBI Genebank AY878328.1 above 0,020 or above 2%.

Table 7: Comparison with data examination of cement

Code	The mean concentration of fresh cement / ml	Overall quality	Results Gene Sequences
2	1.031,4x10^6	Good	Transition
7	799.7 x10^6	Good	Can not be read
8	705.7 x10^6	Good	Transition
13	1418.7 x10^6	Good	Transition
14	1090 x10^6	Good	Transition
17	1075 x10^6	Good	Transition
19	429.4 x10^6	Poor	deletions
D	755.3 x10^6	Good	Transition
H	286.6 x10^6	Poor	Deletions, Transitions
I	1345.9 x10^6	Good	Transition
J	1173.9 x10^6	Good	Transition
L	1290 x10^6	Good	Transition
M	1142.7 x10^6	Good	Transition
N	312.4 x10^6	Poor	Deletions, Transitions

DISCUSSION:

Based on the quantity of isolation cement test results known that almost all of the samples have a good degree of purity that is still within the range of 1.8 to 2.0 and all samples had concentrations above 100 ng / mL. If the DNA purity values below 1.8 indicate the DNA extraction yield there are contaminants in the form of protein compounds. Contamination in the form of protein compounds in the DNA can be caused by the addition of a protease enzyme on DNA isolation protocol. DNA purity value above 2.0 indicates there are contaminants in the form of RNA. This may be due to the addition of the study was not done ribonuclease. According Fatchiyah (2011), nano-drop test result is a value purity DNA at A260 / A280 and the concentration of DNA [13,14].

The results of sequencing all samples alignment homolog with NCBI Genebank database (AY878328.1) obtained their deletion in the code sample 7 from the beginning of the sequence to the end, Sample code H, N, 19 (T - -) bases of DNA polymorphisms to-10080. Characterized by differences in the nucleotide sequence between individuals. Transition (T - C) in the sample code D, H, I, L, M, N, 2, 17, 18 bases to-10044 and transition (G - A) in the sample code H, I, J, N, 2, 8, 13, 14, 17. The deletions affect amino acids that form, because there is a missing nucleotide bases. According Sharma and Sharma (2014) and Griffith *et al*. (1999), transition mutation means a mutation that occurs when pyrimidine bases in DNA nucleotide chain is replaced by another pyrimidine base or purine base is replaced by another purine base [15-19].

The comparison between gene sequences with the results of the concentration data of cement each sample obtained in samples that experienced deletions in the sequence of its gene have a concentration of low cement compared to the group, for example, in a sample of H and N concentrations were low compared to cows FH other males who come from BBIB Singosari. While in the 19 samples had a low concentration also compared with samples 8. The results indicated resources site that this mutation could be related with the trait susceptibility to sperm concentration. It is possible that other genes linked with this molecular genetic markers affected the sperm quality. But the weakness of this study is at least phenotype samples with a low concentration of spermatozoa. Further studies are needed to address reviews these possibilities[20-22].

CONCLUSION:

The results of this study indicate between gene sequences with the results of the concentration data of cement each sample obtained in samples that experienced deletions in the sequence of its gene have a

concentration of low cement and resources site that this mutation could be related with the trait susceptibility to sperm concentration.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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