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Tyrosine Kinase Gene Polymorphisms in Limousin (*Bos taurus*) Bull Correlation with Fresh Semen Qualities

(KORELASI POLIMORFISME GEN TIROSIN KINASE PADA SAPI LIMOUSIN (*BOS TAURUS*) JANTAN TERHADAP KUALITAS SEMEN SEGAR)

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ABSTRACT

Tyrosine kinase (TEK) is the subgroup of protein kinases class. Tyrosine kinase is a protein in spermatozoa plasm that functions as a mediator during spermatozoa penetration to pellucide zone 3 (PZ3) of the ovum. The purpose of this study was to analyse the variation of receptor tyrosine kinase genes related to the fresh semen qualities of Limousin bulls, using Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) method with HindIII restriction enzyme in A/AGCTT restriction site. This study used 20 blood samples of Limousin bull from Singosari National Artificial Insemination Center at Malang. DNA amplification carried out by PCR method using forward primer (TEK_F) 5'-TAGATTGTCGCTTGCCTGGG-3' and reverse primer (TEK_R) 5'-CCTGTGCCGACAGGTTTACT-3'. The data analyses were obtained by counting the total of RFLP DNA fragments as the genetic variation. The correlation between the total of RFLP DNA fragments and the fresh semen qualities were analysed by *Spearman* correlation test with RStudio software. The polymorphic DNA fragments were on 136 bp, 88 bp, 72 bp, and 12 bp range. Monomorphic DNA fragment was on 39 bp range. Correlation coefficient between the RFLP of DNA fragments total with semen pH was -0,158 (negative correlation) with the p-value 0,912; semen volume was -0.105 (negative correlation) with the p-value 0,659, sperm motility was -0,050 (positive correlation) with the p-value 0,831; and sperm concentration was 0.044 (positive correlation) with the p-value 0,852. The RFLP DNA fragment total with some fresh semen evaluation parameters had very low correlation with no significancy because of the p-value >0, 05.

Keywords: hindIII; PCR RFLP;, semen; tyrosine kinase

ABSTRAK

Tirosin kinase (TEK) merupakan salah satu protein pada membran plasma spermatozoa berfungsi sebagai mediator pertemuan antara spermatozoa dengan sel telur pada zona pelusida 3 (ZP3). Penelitian ini bertujuan untuk menganalisis adanya variasi gen tirosin kinase spermatozoa

pada semen segar sapi limousin jantan menggunakan metode *Polymerase Chain Reaction Restriction Fragment Length Polymorphism* (PCR-RFLP) berdasarkan hasil fragmen DNA yang menggunakan enzim restriksi HindIII. Penelitian ini menggunakan 20 sampel darah dan semen segar yang didapat dari Balai Besar Inseminasi Buatan Singosari, Malang. Amplifikasi DNA dilakukan dengan metode PCR menggunakan primer *forward* (TEK_F) 5'-TAGATTGTCGCTTGCCTGGG-3' dan *reverse* (TEK_R) 5'-CCTGTGCCGACAGGTTTACT-3'. Analisis data dilakukan dengan menghitung jumlah fragmen DNA yang terbentuk dan korelasinya terkait kualitas semen segar dianalisis dengan uji *Spearman*. Variasi gen tirosin kinase ditunjukkan dengan terbentuknya fragmen DNA. Fragmen DNA polimorfik berada pada kisaran 136 bp, 88 bp, 72 bp, dan 12 bp, sedangkan fragmen DNA monomorfik berada pada kisaran 39 bp. Koefisien korelasi (r) antara jumlah fragmen DNA pada RFLP dengan pH semen bernilai -0,158 (tidak searah) dengan nilai signifikansi 0,912; volume semen bernilai -0.105 (tidak searah) dengan nilai signifikansi 0,659; motilitas spermatozoa bernilai -0,050 (tidak searah) dengan nilai signifikansi 0,831; dan konsentrasi spermatozoa 0,044 (searah) dengan nilai signifikansi 0,852. Jumlah fragmen DNA pada RFLP dengan beberapa parameter uji kualitas semen segar tersebut memiliki korelasi sangat rendah dan tidak signifikan (signifikansi >0,05).

Kata-kata kunci: hindIII; PCR RFLP; semen; tirosin kinase

INTRODUCTION

Artificial insemination (AI) is a process that inserts semen directly into a female womb, able to increase the cattle productivity and genetic quality. One of factors that successfully determines artificial insemination program is fresh semen qualities. Collected fresh semen must be evaluated to assess the qualities before its processed further in semen freezing process (Susilawati *et al.*, 2019; Yekti *et al*., 2017). One of the cattles used as a semen donor in AI program is limousin bull. Limousin bull has compact body, bright brown hair (beige) colour, body weight from 800-900 kg, and the carcast reaches 50% of body weight (Beran *et al*., 2011; Muada *et al*., 2017).

Nowadays, fresh semen qualities can be evaluated macroscopically (semen volume and pH), and microscopically (sperm motility and concentration) (Muada *et al*., 2017). Fresh semen qualities evaluation are only known to determine the phenotipically qualities in an individual and the genetic potential has not determined yet. Thus, to predict the bull fertility potential must be analysed genetically (in molecular). If the gens that influence the phenotip of sperm qualities are known, the purpose of excellent breed selection with great characteristic can be selected earlier (Hernawati *et al*., 2016; Oktanella *et al*., 2017)

Tyrosine kinase (TEK) is the subgroup of protein kinase class. Tyrosine kinase is a protein in spermatozoa plasm that functions as a mediator between spermatozoa and an ovum in penetrating pellucide zone 3 (PZ3) (Lalancette *et al*., 2006). Ijiri *et al*. (2012) explains that the activity of TEK helps the spermatogenesis process, epididymical maturation, sperm capacitation, acrosomal excogitation, fusion process, and membrane interaction. Tyrosine kinase also helps to stabilize the covalent bound that arranges the protein membrane and activate the transduction signal till the phosporilation process happens. Thus, it can hyperactivate and increase the sperm motility (Madyawati, 2008; Madyawati and Srianto, 2007)

The development of molecular research has used to analyse the genetic variation in DNA level. Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) is one of methods that can be used to detect the genetic variation in population (Hashim and Al-Shuhaib, 2019). The PCR-RFLP method uses DNA marker based on restriction site with endonuclease restriction enzyme. Restriction enzyme can digest and restrict the DNA in certain restriction site to several fragments (Rahayu, 2013).

Based on the background of those problems having been discussed, this study attempts to analyse the variation of tyrosine kinase genes of spermatozoa in limousin bulls' fresh semen with PCR-RFLP, based on amplicon bands pattern resulted by HindIII restriction reaction.

RESEARCH METHODS

Materials

Blood vacutainers, microtube, micropipette, centrifugator, electrophoresis device, microwave, vortex mixer, erlenmeyer tube, PCR tube, UV transluminator Bio-Rad, limousin bulls' blood, Jena Bioscience Blood DNA Preparation Kit (contains RBC lysis

solution, cell lysis solution, protein precipitation solution, washing buffer, dan DNA hydration solution), agarose, TBE (Tris-borate-EDTA) buffer, DNA ladder BenchTop Promega (marker) 1 kb, 100 bp, and 10 bp; PCR mix GoTaq® Master Mixes Promega (berisi dNTPs, $MgCl_2$, Taq polymerase); primer Forward (TEK_F) 5'-TAGATTGTCGCTTGCCTGGG-3', and Reverse (TEK_R) 5'-CCTGTGCCGACAGGTTTACT-3'; HindIII restriction enzyme, and ddH_2O.

Samples Collection

A total of 20 blood-samples were collected from selected bulls of limousin from Artificial Inseminaion Center (Balai Besar Inseminasi Buatan/BBIB) at Singosari, Malang. The samples were collected from the jugularis vein, as much as 3-5 mL of blood volume taken from each bull and put inside vacutainer containing Ethylene Diamine Tetraacetic Acid .

DNA Isolation

Total genome was isolated from the blood samples using the Jena Bioscience Blood DNA Preparation Kit. There are three main steps in DNA isolation: cell lysis, separation of DNA from solid materials (such as cellulose and proteins), and DNA purification (Green and Sambrook, 2018).

DNA Amplification and RFLP

The DNA amplification used forward and reverse primer with 10 pmol concentration each as much as 1.5 µL; DNA isolate 4 µL; ddH_2O ad. 24 µL. PCR technique used thermal cycler Bio-Rad with predenaturation 94°C 2 min; denaturation 94°C 30 s; annealing 58-61°C 30 s; elongation 72°C 1 min; and post elongation 72°C 7 min (Hernawati *et al*., 2021). PCR products were restricted with HindIII restriction enzyme. The reagents that used are PCR DNA product as much as 4 µl; nuclease free water (NFW) 17,2 µl; buffer tango 3 µl; and HindIII 0,8 µl. The RFLP is incubated with 37°C temperature in one night.

Data Analysis

The data analysis was obtained by counting the total of RFLP DNA fragments as the genetic variation. The correlation between the total of RFLP DNA fragments and the fresh semen qualities were analysed by Spearman correlation test with RStudio software (RStudio Team, 2015).

RESULTS AND DISCUSSION

DNA Isolation

The total DNA were isolated from 20 limousin (*Bos taurus*) bulls whole-blood samples by Jena Bioscience Blood DNA Preparation Kit. The total DNA templates were tested quantitatively using a Nano-200 Micro-nucleic acid spectrophotometer machine on 230 nm, 260 nm and 280 nm wavelengths (λ).

Table 1. DNA samples concentration and purity test result.

No.	Concentration (ng/µL)	Purity (260/230 nm)	Purity (260/280 nm)
1	29.22	1.19	1.09
2	32.48	0.92	1.05
3	18.78	0.73	0.96
4	33.48	0.55	0.82
5	40.95	0.68	0.80
6	15.86	0.72	1.10
7	29.02	0.85	1.06
8	32.32	0.51	0.75
9	41.90	0.71	0.75
10	17.93	0.55	0.81
11	39.39	0.77	0.80
12	69.03	0.83	0.71
13	43.66	0.53	0.72
14	28.91	0.41	0.69
15	43.76	0.67	0.73
16	51.42	0.72	0.69
17	19.94	0.46	0.78
18	78.19	0.65	0.70
19	13.40	0.52	0.71
20	13.62	0.67	0.85

Note: DNA purity in λ 260/230 nm = 2-2.2; DNA purity in λ 260/230 nm = 1.8-2 (Green and Sambrook. 2018).

Abdel-Latif and Osman (2017) explains that DNA quantity test with spectrophotometer can be calculated accurately through the absorption of ultraviolet light. Double fragment of DNA can absorb the UV light in wavelength (λ) 260 nm. the protein contaminants can absorb the UV light in wavelength (λ) 280 nm. and the polysaccharide or another chemical content contaminants can absorb the UV light in (λ) 230 nm (Green and Sambrook. 2018).

The DNA purity level through protein contaminants are between 1.8-2.0; while the DNA purity level through polysaccharide contaminants are between 2.0-2.2. When the DNA purity level is below 1.8 for λ 260/280 nm. it means that the sample still contains protein contaminants. When the DNA purity level is below 2.0 for λ 260/230 nm. it means that the sample still contains polysaccaride or another chemical content contaminant (Boesenberg-Smith *et al*.. 2012).

The study concludes that the DNA purities in all samples were below the range levels. It shows that all samples probably still contained protein. but all samples showed the ideal DNA concentration which was 10 ng/µL and it is good for amplification process (Abdel-Latif and Osman. 2017; Boesenberg-Smith *et al*.. 2012). The result of total qualities DNA isolation which was tested by using 1% agarose gel electrophoresis obtained a total DNA band with fragment size >10.000 bp which is presented in Figure 1.

DNA Amplification and RFLP

The result of DNA isolation samples were continued to the amplification process with Polymerase Chain Reaction (PCR) by thermal cycler Bio-Rad and the PCR program can be seen in Table 3. The primers used to amplify the TEK gene were taken from genebank with the sequence number NM_1739642. A pair of primers used to perform TEK gene amplification in limousin bulls are shown in Table 2.

Table 2. Tyrosine kinase gene primary oligonucleotide

Primer	Oligonucleotide Sequence
Forward (TEK_F)	5'-TAGATTGTCGCTTGCCTGGG-3'
Reverse (TEK_R)	5'-CCTGTGCCGACAGGTTTACT-3'

Table 3. Polymerase Chain Reaction programs for amplification

Steps	Time	Temperature
Predenaturation	3 m	94°C
Denaturation	30 s	94°C
Annealing	30 s	58-61°C
Extension	1 m	72°C
Post Extension	7 m	72°C

The amplification process for approximately 90 minutes produces a product which was then passed through a qualitative agarose electrophoresis test of 1.8%. Electrophoresis results can be seen in Figure 2.

Hernawati *et al*. (2021) explains that the desired target band of PCR products using designed primers is 302 bp. The results of visualization on the PCR product showed a ribbon with a 302 bp fragment size according to the target based on the primary design. DNA PCR products were restricted with HindIII restriction enzyme. The RFLP products were

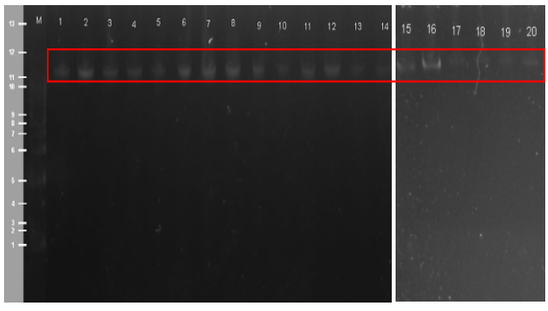

Figure 1. DNA isolation electrophoresis visualization in agarose gel 1% (M = marker; 1-20 = total DNA samples)

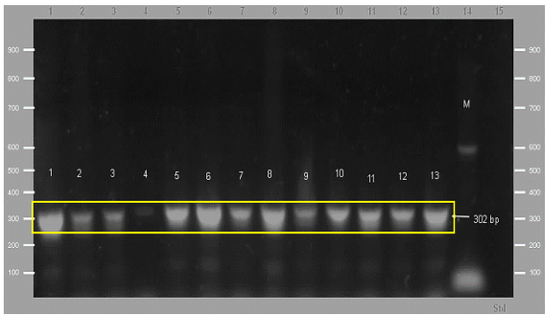

Figure 2. DNA PCR products electrophoresis visualization in agarose gel 1.8% (M = marker; 1-13 = DNA PCR products)

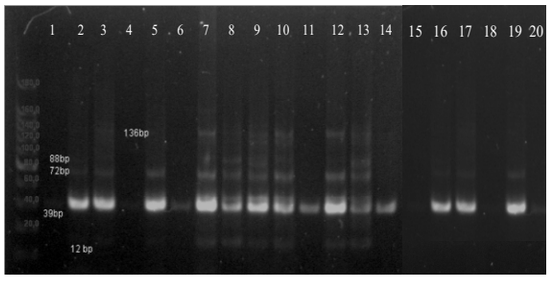

Figure 3. RFLP electrophoresis visualization in agarose gel 3% (1-20 = RFLP samples; monomorphic fragment no. 3 = 39 bp; polimorphic fragments no. 1 = 136 bp. 2 = 88 bp. 4 = 72 bp. 5 = 12 bp.

shown in electrophoresis with 3% agarose gel and 10 bp DNA ladder. The result was visualized in UV transluminator Bio-Rad (Figure 3).

Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) is used to analyse the polymorphism of genome in organism with specific restriction enzyme (Hashim and Al-Shuhaib. 2019). This study uses HindIII restriction enzyme (which is produced by *Haemophilus influenza* Rd) restricts the DNA A/AGCTT sequence (Mo *et al*.. 2004). The restriction enzyme will break the DNA chains and make easy recognize the specific DNA sequences (Schütte *et al*.. 2008).

There are two patterns produced by the DNA fragment restriction: polymorphic (the DNA fragment restricted in the different sizes but partly does not appear in all samples) and monomorphic (the DNA fragment restricted in the same size and appears in all samples) (Hashim and Al-Shuhaib. 2019). The monomorphic DNA fragment is on 39 bp range. The polymorphic DNA fragments are on 136 bp. 88 bp. 72 bp. and 12 bp.

Data Analysis

The fresh semen qualities data was obtained from National Artificial Inswmination Center (Balai Besar Inseminasi Buatan/BBIB) at Singosari. Malang with five5 times data sampling. which was done on January-May 2019. The fresh semen qualities include semen pH. semen volume. sperm motility. and sperm concentration. After the data qualities obtained. it was correlated with the total of RFLP DNA fragments to know the correlation of the variables. The fresh semen qualities data and the total of RFLP DNA fragments were shown in Table 4.

Table 4. The fresh semen qualities data and the total of Restriction Fragment Length Polymorphism/RFLP DNA fragments

No.	Semen pH	Semen volume (mL)	Sperm motility (%)	Sperm concentration (x10^6/mL)	RFLP DNA fragments total
1	6.3	6.8	69.90%	804.6	3
2	6.8	6.9	88.50%	967	3
3	6.9	6.4	90.60%	1.038.20	4
4	6.4	5.9	63.20%	854	1
5	6.4	7.4	59.20%	995	3
6	6.8	6.7	88.30%	873.2	1
7	6.8	7.7	88.80%	646.2	5
8	6.2	5.5	65.50%	1.234.80	5
9	6.2	5.4	55.00%	1.360.40	5
10	6.8	4.5	85.80%	1.241.80	5
11	6.2	6.2	89.70%	808.2	1
12	6.2	4.9	86.50%	927.8	4
13	6.3	5.2	65.30%	743.8	5
14	6.7	5.4	90.60%	995	1
15	6.8	5.4	83.50%	1.160.60	1
16	6.8	3.1	87.70%	1.088.20	3
17	6.8	5.6	90.70%	566.4	3
18	6.4	3.6	60.30%	831.8	1
19	6.6	7	29.20%	754	3
20	6.2	7.4	61.90%	1.632.00	1

Normality Test Data

The data normality test used to know the data result is normally or non normally distributed (Ghasemi and Zahediasl. 2012). The normal distribution data shows the significancy >0.05; if the significancy <0.05. the data is non normally distributed. The normal distribution data will be analysed with parametric statistical test. but if the data is non normally distributed will be analysed with non parametric statistical test (such as *Spearman* test). Normality data test uses Shapiro-Wilk by RStudio software with the significancy 0.05. Shapiro-Wilk is used to test the normality data belows 50 samples and gives accurate decision (Hanusz *et al*.. 2016). The result of data normality is shown in Table 5.

Table 5. Normality data result

Data	Significancy
RFLP DNA fragments amount	0.00221
Semen pH	0.00184
Semen volume (mL)	0.466
Sperm motility (%)	0.00235
Sperm concentration (10^6/mL)	0.513

Based on the data normality result (Table 5). it can be concluded that if the significancy >0.05 the data is normally distributed. If the significancy <0.05 the data is non normally distributed. The correlation analysis will use non parametric statistical test. which is *Spearman* correlation test using RStudio software (RStudio Team. 2015).

Correlation Test Between the DNA RFLP Fragments Total with Fresh Semen Qualities

The correlation test in this study used Spearman by RStudio software. Sperman test is one of correlation methods used to measure the correlation between two variables. The two variables have not to be normally distributed data. The result of Spearman correlation test is shown in Table 6.

In correlation test between DNA RFLP fragments total with semen pH (Table 6) showed r = -0.0263 which meant the correlation was very low (Myers and Sirois. 2004; Sedgwick. 2014) with significancy 0.912. Negative correlation means that as much as DNA RFLP fragments formed. the semen pH will decrease. Inversely. as few as DNA RFLP fragments formed. the semen pH will increase.

In correlation test between DNA RFLP fragments total with semen volume (Table 6) showed r = -0.105 which meant the correlation was very low (Myers and Sirois. 2004; Sedgwick. 2014) with significancy 0.659. Negative correlation means that as much as DNA RFLP formed. the semen volume will decrease. Inversely. as few as DNA RFLP formed. the semen volume will increase. In correlation test between DNA RFLP fragments total with sperm motility (Table 6) showed r = -0.050 which meant the correlation was very low) with significancy 0.831. Negative correlation means that as much as DNA RFLP formed. the sperm motility will decrease. Inversely. as few as DNA RFLP formed. the sperm motility will increase. In correlation test between DNA RFLP fragments total with sperm concentration (Table 6) showed r = 0.044 which meant the correlation was very low) with significancy 0.852. Positive correlation means that as much as DNA RFLP formed. the sperm motility will increase. and as few as DNA RFLP formed. the sperm motility will decrease.

Correlation between DNA RFLP fragments total with fresh semen pH showed that as few as DNA RFLP fragments total formed. the fresh semen pH would increase. It means that as few as polymorphism in tyrosine kinase gen make fresh semen pH increase. (Madyawati and Srianto. 2007) explains that optimum pH of tyrosine kinase is 7 and tyrosine kinase will maximally work in this pH. The significancy is 0.912 (>0.05) and shows the correlation is not significant (Janie. 2012).

Table 6. Spearman correlation test between the DNA Restriction Fragment Length Polymorphism RFLP fragments total with fresh semen qualities result

Correlation	Correlation coefficient (r)	Correlation interpretation	Significancy (p-value)
DNA RFLP fragments total with semen pH	-0.0263	Very low (negative correlation)	0.912
DNA RFLP fragments total with semen volume (mL)	-0.105	Very low (negative correlation)	0.659
DNA RFLP fragments total with sperm motility (%)	-0.050	Very low (negative correlation)	0.831
DNA RFLP fragments total with sperm concentration (10^6/mL)	0.044	Very low (positive correlation)	0.852

Correlation between DNA RFLP fragments total with fresh semen volume showed that as much as DNA RFLP fragments total formed. the fresh semen volume would decrease. Sperm volume contains membrane liquid and some chemical components (such as cholesterol. ion. etc) that can activate capacitation process (Naz and Rajesh. 2004). As much as tyrosine kinase activity can decrease the semen volume that influence capacitation process. Inversely. as few as tyrosine kinase activity can increase the semen volume that influence capacitation process. The signifincany is 0.659 (>0.05) and shows the correlation is not significant (Janie. 2012).

Correlation between DNA RFLP fragments total with sperm motility showed that as much as DNA RFLP fragments total formed. the sperm motility would decrease. Hernawati *et al*. (2021) also explains that tyrosine kinase gene sequence mutation increasing can decrease sperm motility. It shows that polymorphisms in tyrosine kinase gene can decrease the phosporilation process and decrease the activation of sperm motility. Tyrosine kinase is inisiated by tyrosine kinase receptor (Ijiri *et al*.. 2012). When the extracellular ligand binds with the receptor. the tyrosine kinase receptor will be activated and do the phosporilation process. Phosporilation product will trigger the sperm motility. The signifincany is 0.831 (>0.05) and shows the correlation is not significant (Janie. 2012).

Correlation between DNA RFLP fragments total with sperm concentration showed that as much as DNA RFLP fragments total formed. the sperm motility would increase. While Hernawati *et al*. (2021) also explains that tyrosine kinase gene sequence mutation increasing can decrease sperm concentration. One of tyrosine kinase functions is activating the sperm capacitation. Sperm concentration is one of components that can activate the capacitation and show the sperm total ($x10^6$) in 1 mL (Atiq *et al*.. 2011). As much as sperm concentration can normally activate the capacitation process. Inversely. as few as sperm concentration can decrease the activation of capacitation process. Thus. the polymorphisms in tyrosine kinase gene can decrease the sperm concentration which can decrease the capacitation activation process. The signifincany is 0.852 (>0.05) and shows the correlation is not significant (Janie. 2012).

CONCLUSION

The polymorphisme of tyrosine kinase gene can be detected with PCR RFLP with HindIII enzyme restriction. The polymorphic DNA fragments were on 136 bp. 88 bp. 72 bp. and 12 bp range. Monomorphic DNA fragment was on 39 bp range. Correlation coefficient between the RFLP DNA fragments total with semen pH was -0.158 (negative correlation) with the p-value 0.912; semen volume was -0.105 (negative correlation) with the p-value 0.659. sperm motility was -0.050 (positive correlation) with the p-value 0.831; and sperm concentration was 0.044 (positive correlation) with the p-value 0.852. The RFLP DNA fragment total with some fresh semen evaluation parameters had very low correlation.

SUGGESTION

The next research can use longer target of PCR products to know the polymorphisme in another nucleotide sequence.

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REFFERENCES

Abdel-Latif A. Osman G. 2017. Comparison of three genomic DNA extraction methods to obtain high DNA quality from maize. *Plant Methods* 13(1): 1–9.

Atiq N. Ullah N. Andrabi SMH. Akhter S. 2011. Comparison of photometer with improved neubauer hemocytometer and makler counting chamber for sperm concentration measurement in cattle. *Pak Vet J* 31(1): 83–84.

Beran J. Stádník L. Ducháček J. Toušová R. Louda F. Štolc L. 2011. Effect of bulls' breed. age and body condition score on quantitative and qualitative traits of their semen. *Acta Univ Agric et Silvic Mendel Brun* 59(6): 37–44.

Boesenberg-Smith KA. Pessarakli MM. Wolk DM. 2012. Assessment of DNA yield and purity: An overlooked detail of PCR troubleshooting. *Clinical Microbiology Newsletter* 34(1): 1–6.

Ghasemi A. Zahediasl S. 2012. Normality tests for statistical analysis: A guide for non-statisticians. *International Journal of Endocrinology and Metabolism* 10(2): 486.

Green MR. Sambrook J. 2018. Isolation and quantification of DNA. *Cold Spring Harbor Protocols* 2018(6). pdb-top093336.

Hanusz Z. Tarasinska J. Zielinski W. 2016. Shapiro–Wilk test with known mean. *REVSTAT-Statistical Journal* 14(1): 89–100.

Hashim HO. Al-Shuhaib MBS. 2019. Exploring the potential and limitations of PCR-RFLP and PCR-SSCP for SNP detection: A review. *Journal of Applied Biotechnology Reports* 6(4): 137–144.

Hernawati T. Mulyati S. Oktanella Y. 2016. Specific Protein Sperm Membrane Supplementation on Freezing Medium Maintain Post-Thawed Bull Sperm Quality. *Sphinx Knowledge House* 9(12){ 576–587.

HernawatiT. Oktanella Y. Dhaneswari SA. 2021. Tyrosine Kinase Gene Polymorphisms Associate with Fresh Semen Quality from Dairy Bull Friesian Holstein. *Iraqi Journal of Agricultural Sciences* 52(4): 1050–1057.

Ijiri TW. Mahbub Hasan AKM. Sato K. 2012. Protein-tyrosine kinase signaling in the biological functions associated with sperm. *Journal of Signal Transduction*. 2012 | Article ID 181560 | https://doi.org/10.1155/2012/181560

Janie DNA. 2012. *Statistik deskriptif & regresi linier berganda dengan* SPSS. Semarang. Semarang University Press.

Lalancette C. Faure RL. Leclerc P. 2006. Identification of the proteins present in the bull sperm cytosolic fraction enriched in tyrosine kinase activity: A proteomic approach. *Proteomics* 6(16): 4523–4540.

Madyawati SP. 2008. Suplementasi tirosin kinase spermatozoa sapi Friesian Holstein (FH) terhadap kualitas semen beku [*Disertasi*]. Surabaya. Universitas Airlangga.

Madyawati SP. Srianto P. 2007. Optimasi Aktivitas Tyrosin Kinase Hasil Isolasi dari Spermatozoa Sapi Perah Frisian Holstein (FH). *Media Kedokteran Hewan 23*(3): http://journal.unair.ac.id/MKH@the-optimation-of-tyrosine-kinase-activity-from-sperm-isolation-of-friesian-holstein-cow-article-2662-media-1-category-1.html

Mo X.-Y. Cao C-K. Xu F-H. Liu M-Y. Li M-X. Qin Y-J.. Zhou Q. Zhang Y-Y. Deng H-. 2004. Lack of association between the HindIII RFLP of the osteocalcin (BGP) gene and bone mineral density (BMD) in healthy pre-and postmenopausal Chinese women. *Journal of Bone and Mineral Metabolism* 22(3){ 264–269.

Muada DB. Paputungan U. Hendrik M J. Turangan SH. 2017. Karakteristik semen segar sapi bangsa limousin dan simmental di balai inseminasi buatan lembang. *Zootec* 37(2): 360–369.

Myers L. Sirois MJ. 2004. Spearman correlation coefficients. differences between. *Encyclopedia of Statistical Sciences 12*. DOI:10.1002/0471667196.ESS5050

Corpus ID: 117033393

Naz RK. Rajesh PB. 2004. Role of tyrosine phosphorylation in sperm capacitation/ acrosome reaction. *Reproductive Biology and Endocrinology*. 2(1): 1–12.

Oktanella Y. Firmawati A. Hendrawan VF. Wulansari D. 2017. Comparison Between Oviduct Fluid Protein and Oviductal Epithelia Cell As Supplements In Capacitation Media To Improve Sheep's Spermatozoa Quality. *International Journal of ChemTech Research* 10(4): 565–571.

Rahayu S. 2013. Analisis Polimorfisme Gen Growth Differentiation Factor 9 (GDF-9) dan Hubungannya dengan Keberhasilan Inseminasi Buatan pada Sapi PO. *Biotropika: Journal of Tropical Biology*. 1(3): 95–100.

Schütte UM. Abdo Z. Bent SJ. Shyu C. Williams CJ. Pierson JD. Forney LJ. 2008. Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. *Applied Microbiology and Biotechnology* 80(3){ 365–380.

Sedgwick P. 2014. Spearman's rank correlation coefficient. *BMJ* 2014 Nov 28;349:g7327. doi: 10.1136/bmj.g7327

Susilawati T. Mahfud A. Isnaini N. Yekti APA. Huda AN. Satria AT. 2019. The comparison of artificial insemination success between unsexed and sexed sperm in Ongole Crossbred cattle. *IOP Conference Series: Earth and Environmental Science* 387(1): 012010.

RStudio Team. 2015. RStudio: Integrated development for R. *RStudio. Inc.. Boston. MA URL Http://Www. Rstudio. Com*. 42(14). 84.

Yekti APA. Susilawati T. Ihsan MN. Wahyuningsih S. 2017. *Fisiologi Reproduksi Ternak: Dasar Manajemen Reproduksi*. Malang. Universitas Brawijaya Press.