DOCTORAL THESIS

Study on genetic markers in the bovine *IARS* and *FOXP3* genes associated with reproductive performance in cattle

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DEDICATION

I dedicate this thesis to my beloved father and mother and to the soul of my departed grandfather and grandmother, who gave me an inspiration of learning and taught me the value of honesty, perseverance and resolving.

ABSTRACT

Infertility in cows is a significant global concern associated with multiple unidentified factors, including those relating the maternal body, bull semen, nutrition, and the environment. Among the different maternal factors, genetic disorders and detrimental traits linked to inherent gene and chromosomal abnormalities also compromise reproductive efficiency in cattle. Besides, there are some biochemical markers that affect maternal fertility, including anti-Müllerian hormone (AMH), a glycoprotein belonging to the transforming growth factor-beta superfamily and secreted by ovarian granulosa cells primarily from pre-antral and early antral follicles of females, and serum amyloid A (SAA), one of the most reliable acute phase proteins primarily produced by the liver via stimulus of inflammatory cytokines. AMH and SAA are recently demonstrated to be associated with reproductive performance of beef and dairy cows. In this study, bovine isoleucyl-tRNA synthetase (IARS) disorder (Chapter 1) and bovine Forkhead Box P3 (FOXP3) alteration in combination with AMH and SAA concentrations (Chapter 2) were investigated in order to demonstrate the relationship between these maternal factors and cattle fertility.

Chapter 1: Bovine IARS disorder, a major cause of weak calf syndrome, is caused by a homozygous missense (c.235G>C) mutation in the bovine *IARS* gene of JB cattle, which was identified in 2013. However, the extent to which the carrier rate has changed at Kagoshima prefecture, Japan, and whether the carrier status is associated with any clinical or reproductive

problems, have yet to be ascertained. In this study, using a real-time polymerase chain reaction-based genotyping assay, we determined the carrier rate in a regional JB cow population at Kagoshima prefecture. Comparative analyses were performed on the metabolic profile test (MPT) results and reproductive performance data obtained for heterozygous carrier and homozygous wild-type cows. In 2009 and 2018, DNA samples were collected from 130 and 462 clinically healthy JB cows, respectively, in Kagoshima prefecture. Therefore, MPT results and reproductive performance data were evaluated for 62 cows, comprising four heterozygous carriers and 58 wild-type cows. Genotyping revealed that the carrier rate was 6.9% in 2009 and 1.5% in 2018, the difference of which was statistically significant ($P < 0.005$). There were no statistically significant differences between the carrier and wild-type cows with respect to either MPT results or reproductive performance, indicating that the carrier cows have necessary IARS activity to maintain minimal health and reproductive potential.

Chapter 2: Immune adaptation plays an essential role in determining pregnancy, which has been shown to be dependent on sufficient immunological tolerance mediated by FOXP3+ regulatory T cells. Recently, an X-linked maternal single-nucleotide polymorphism (SNP), located 2175 base pairs up-stream of the start codon in the bovine *FOXP3* gene (NC_037357.1: g.87298881A>G, rs135720414), was identified in JB cows (*Bos taurus*) in association with recurrent infertility. However, with the exception of JB cows, the frequency of this SNP has yet to be studied in other cow populations. The objective of this study was to evaluate the

frequency of this SNP in different cow breeds. Between 2018 and 2021, a total of 809 DNA samples were obtained from 581 JB, 73 Holstein Friesian (HF: *B. taurus*), 125 Korean Hanwoo (KH: *B. taurus coreanae*), and 30 Indonesian Madura (IM: a crossbreed between *B. indicus* and *B. javanicus*) cows, which were genotyped using a TaqMan probe-based real-time polymerase chain reaction assay designed in this study. The G allele frequency was found to be relatively high in local IM (0.700), moderate in dairy HF (0.466), and low in beef JB (0.250) and KH (0.112) cows, with differences in the frequencies between each group being statistically significant ($P < 0.005$) using Fisher's exact test. The results obtained in this study indicate that the G allele frequencies of the identified SNP differ markedly in different breeds of taurine and indicine cattle. Given these findings, it would thus be important to evaluate the relationships between high frequencies of the G allele and infertility in different breeds.

Moreover, in this study using HF cows, AMH concentration in parous cows with SNP A/A was significantly higher ($P < 0.05$) than that in parous cows with A/G and G/G, indicating that A/A parous cows may have a higher fertility than other genotypes of parous cows. There was not a similar observation in HF heifers. In the range of low SAA concentration, A/A parous cows tended to be more abundant than other types of parous cows. Based on these observations, parous HF cows carrying the G allele may be susceptible to infections, leading to subsequent inflammatory conditions. The inflammatory conditions possibly cause decreased concentration of AMH resulting in infertility.

In conclusion, in this study (Chapter 1), genotyping of the JB population in Kagoshima prefecture revealed that there remains a risk of producing both carriers and affected cows for IARS gene mutation. There was no statistically significant difference in the MPT results or reproductive performance of carrier and wild-type cows, indicating that the carrier cows have necessary IARS activity to maintain minimal health and reproductive potential. By detecting anomalies such as underlying ARS deficiencies in cattle populations, these types of analyses could also be beneficially applied to facilitate maintenance of the equality of cow herds. Furthermore, on the basis of the results obtained in this study (Chapter 2), it was established that current frequencies of the detrimental G allele of the SNP in the upstream of the bovine *FOXP3* gene are particularly high in local populations of IM cows compared with those of three other assessed breeds, among which, G allele frequencies are low in KH and JB cows, and moderate in HF dairy cows. Given these findings, it would thus be important to evaluate the relationships between high frequencies of the G allele and infertility in different breeds. Given that the moderate to high G allele frequencies in IM and HF cows are plausibly associated with the infertility of these breeds, it would be interesting to evaluate the relationship between the high frequency of the risk-type G allele and infertility. In this regard, the genotyping assay developed in this study could make a notable contribution to surveying the bovine populations. In addition, on the basis of results regarding AMH and SAA, parous HF cows with wild-type A/A genotype had significantly higher AMH concentration than that in

HF cows with other genotypes (G/A and A/A). Inflammatory condition indicated by an increased SAA concentration may potentially decrease AMH concentration particularly in G/A and A/A cows. These results suggest that the G allele may be associated with infertility in HF cows.

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FREQUENTLY USED ABBREVIATION

µl	-	microlitre
µmol/l	-	micromoles per litre
3-HB	-	3-hydroxybutyrate
Alb	-	albumin
AMH	-	anti-Mullerian hormone
ARS	-	aminoacyl-tRNA synthetases
AST	-	aspartate aminotransferase
BUN	-	blood urea nitrogen
c.DNA	-	complementary deoxyribonucleic acid
Ca	-	calcium
DNA	-	deoxyribonucleic acid
FAM	-	6-carboxyfluorescein
FFA	-	free fatty acid
FOXP3	-	forkhead box p3
FTA	-	flinders technology associates
g	-	grams
g.	-	genomic sequence
GGT	-	γ-glutamyl transferase
Glu	-	glucose
GWAS	-	genome wide association studies
HF	-	Holstein friesian
IARS	-	isoleucyl-tRNA synthetase
IM	-	Indonesian madura
iP	-	inorganic phosphorus

JB	-	Japanese black
KH	-	korean hanwoo
Mg	-	magnesium
mg/dl	-	milligrams per deciliter
mg/l	-	milligrams per litre
MGB	-	minor groove binder
mmol/l	-	millimoles per litre
MPT	-	metabolic profile test
NA	-	not applicable
NFQ	-	non-fluorescent quencher
OMIA	-	online mendelian inheritance of animal
p.	-	protein sequence
PCR	-	polymerase chain reaction
rs	-	reference snp
SAA	-	serum amyloid A
SNP	-	single nucleotide polymorphism
T-Cho	-	total cholesterol
TG	-	triglyceride
Tm	-	melting temperature
TP	-	total protein
Treg	-	regulatory T cell
tRNA	-	transfer ribonucleic acid
U/l	-	units per litre
VIC	-	6-carboxyrhodamine

CHAPTER 1

Metabolic profile and reproductive performance of Japanese Black cows associated with bovine isoleucyl-tRNA synthetase (*IARS*) gene mutation

The above-titled work originally appeared in "*Journal of Veterinary Medical Science* (Islam et al., 2021)" as: **Carrier rate of the c.235G>C mutation in the bovine isoleucyl-tRNA synthetase (*IARS*) gene of Japanese Black cows at Kagoshima prefecture, Japan, and analysis of the metabolic profiling and reproductive performance of heterozygous cows** authored by:

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1.1. ABSTRACT

Bovine isoleucyl-tRNA synthetase (IARS) disorder, a major cause of weak calf syndrome, is caused by a homozygous missense (c.235G>C) mutation in the bovine *IARS* gene of Japanese Black (JB) cattle, which was identified in 2013. However, the extent to which the carrier rate has changed at Kagoshima prefecture, Japan, and whether the carrier status is associated with any clinical or reproductive problems, have yet to be ascertained. In this study, using a real-time polymerase chain reaction based genotyping assay, we determined the carrier rate in a regional JB cow population at Kagoshima prefecture. Comparative analyses were performed on the metabolic profile test (MPT) results and reproductive performance data obtained for heterozygous carrier and homozygous wild-type cows. In 2009 and 2018, DNA samples were collected from 130 and 462 clinically healthy JB cows, respectively, in Kagoshima prefecture. MPT results and reproductive performance data were evaluated for 62 cows, comprising four heterozygous carriers and 58 wild-type cows. Genotyping revealed that the carrier rate was 6.9% in 2009 and 1.5% in 2018, the difference of which was statistically significant ($P<0.005$). There were no statistically significant differences between the carrier and wild-type cows with respect to either MPT results or reproductive performance, indicating that the carrier cows have necessary IARS activity to maintain minimal health and reproductive potential.

1.2. INTRODUCTION

Aminoacyl-tRNA synthetases (ARSs), which are highly conserved as ubiquitously expressed house-keeping enzymes, play an important role in protein synthesis by coupling amino acids with their corresponding tRNAs and are characterized by the presence of a specific anticodon triplet, which is central to the transfer of hereditary information via the genetic code (Schimmel, 1987; Park et al., 2008; Yao and Fox, 2013). In humans, the etiology of numerous genetic diseases, including autoimmune diseases, cancer, diabetes, myopathies, liver disease and neurodegenerative disorders, are associated with specific mutations in ARS genes (Antonellis and Green, 2008; Park et al., 2008; Fuchs et al., 2019).

In veterinary medicine, a disorder associated with mutations of the isoleucyl-tRNA synthetase (IARS), a member of the class-1 ARS family, was first reported in 2013 in Japanese Black (JB) cattle as a major cause of weak calf syndrome, the causative mutation of which was identified as a recessive missense mutation c.235G>C (p.V79L) in the bovine *IARS* gene (Hirano et al., 2013). Individuals homozygous for this bovine *IARS* mutation are characterized by intrauterine growth retardation, neonatal weakness, anemia with bone marrow dysfunction, prenatal death and increased mortality prior to 3 months of age (Hirano et al., 2013; Hirano et al., 2016; Hirano et al., 2017). Given these multiple health problems, IARS disorder may have contributed to substantial economic losses in the JB cattle industry due to low reproduction, feed inefficiency, poor carcass characteristics and pre- and postnatal calf mortality in the

affected individuals (Hirano et al., 2016). Therefore, it is of considerable importance to evaluate the impact of IARS disorder and establish prevention measures by determining the carrier rate in the JB cow population.

Furthermore, although carrier cows ostensibly appear to be as healthy as wild-type cows that do not carry the *IARS* mutation, it has yet to be established whether the heterozygous carriers of IARS disorder are predisposed to any clinical or reproductive problems. In this regard, the metabolic profile test (MPT) is recognized as a useful procedure for assessing the performance of clinically healthy animals, in order to identify hidden illnesses and deficiencies, including low production, long calving intervals and other subclinical reproductive problems not only in dairy cows (Payne et al., 1970) but also in beef cows (Watanabe et al., 2013a; Watanabe et al., 2013b; Watanabe et al., 2014; Shinya et al., 2020). To date, the metabolic effects of the heterozygous *IARS* mutation have yet to be investigated in carrier cows. However, performing genotyping in conjunction with the MPT might enable us to identify important differences in the blood parameters and reproductive performance of carrier and wild-type cows.

In this study, we used a real-time polymerase chain reaction (RT-PCR)-based genotyping assay to determine the carrier rate in the JB cow population born before identification of the *IARS* mutation and performed comparative analyses of MPT results and reproductive performance data obtained for heterozygous carrier and wild-type cows raised on same farm.

1.3. MATERIALS AND METHODS

Blood sampling

The experiments conducted in this study were performed in accordance with the guidelines regulating animal use and ethics at Kagoshima University (Permit Number: VM15041). In 2009 and 2018, blood samples were collected from 130 and 462 clinically healthy JB cows, respectively, all born before identification of the *IARS* mutation and raised on several private JB cattle farms in Kagoshima prefecture, Japan. The 462 cows surveyed in 2018 were born between 2006 and 2010. Blood samples were obtained by jugular venipuncture, collected in plain vacuum tubes and transported to the laboratory immediately after collection using a cool box. Serum was separated from the blood within 2 hr after collection and stored at -20°C until used for biochemical analyses in the MPT. A few drops of the centrifuged whole blood obtained after serum separation were spotted onto Flinders Technology Associates filter papers (FTA card; Whatman International Ltd., Piscataway, NJ, U.S.A.) and stored in a refrigerator (4°C) until used for genotyping.

Genotyping

The primers and TaqMan minor groove binder (MGB) probes used for the RT-PCR assay, which were designed based on the sequence of bovine *IARS* gene exon 2 (NCBI Reference Sequence NC_037335.1), are listed in Table 3. These primers and probes, each of which were

linked to a fluorescent reporter dye (6-carboxyrhodamine or 6-carboxyfluorescein) at the 5′-end and a non-fluorescent quencher dye at the 3′-end, were synthesized by a commercial company (Applied Biosystems, Foster City, CA, U.S.A.). As DNA templates, we used DNA extracted from discs punched out of the blood-impregnated FTA cards, as previously described (Mizukami et al., 2011). RT-PCR amplifications were carried out in a final volume of 5 μl consisting of 2× PCR master mix (TaqMan GTXpress Master Mix; Applied Biosystems), 80× genotyping assay mix (TaqMan SNP Genotyping Assays; Applied Biosystems) containing the specific primers, TaqMan MGB probes and template DNA. A negative control containing nuclease-free water instead of template DNA was included in each run. The cycling conditions consisted of 20 sec at 95°C, followed by 50 cycles of 3 sec at 95°C and 20 sec at 60°C. The holding stage after PCR was carried out at 25°C for 30 sec. The data obtained were analyzed using StepOne version 2.3 (Applied Biosystems). Several control DNA samples obtained from healthy cows were used to evaluate the genotyping assay after the genotypes had been confirmed by Sanger sequencing (Hokkaido System Science Co., Ltd., Sapporo, Japan) using the specific primers shown in Table 3.

Analyses of serum biochemicals (MPT) and reproductive and developmental parameters

The following biochemical parameters were measured in the serum samples collected from 462 clinically healthy JB cows in 2018 using a Labospect 7080 autoanalyzer (Hitachi

Ltd., Tokyo, Japan): total protein (TP), albumin (Alb), glucose (Glu), blood urea nitrogen (BUN), triglyceride (TG), total cholesterol (T-Cho), free fatty acid (FFA), 3-hydroxybutyrate (3-HB), calcium (Ca), inorganic phosphorus (iP) and magnesium (Mg) concentrations, and aspartate aminotransferase (AST) and γ-glutamyl transferase (GGT) activities. The Alb to globulin (A/G) ratio was calculated based on TP and Alb concentrations. Among the 462 clinically healthy cows, 62 from the most homogenous herd (mean age: 11.5 years) raised on the same cattle farm were selected to evaluate the MPT results and reproductive and developmental parameters (number of live births, birth weight, daily weight gain and frequency and cost of treatments) in homozygous wild-type (n = 58) and heterozygous carrier (n = 4) cows, which were determined using the genotyping assay.

Statistical analysis

The difference of the carrier rates between 2009 and 2018 was statistically analyzed using Fisher's exact test. Data of the MPT results and reproductive and developmental parameters are presented as the mean ± standard deviation. Statistical analyses for these data were performed using the Mann-Whitney *U* test. *P* values less than 0.05 were considered to indicate a statistically significant difference.

1.4. RESULTS

Genotyping

Using the RT-PCR method, we genotyped a total of 592 JB cows from Kagoshima prefecture, Japan: 130 samples collected in 2009 and 462 samples collected in 2018. Among the samples collected in 2009 and 2018, we detected nine (6.9%) and seven (1.5%) heterozygous carriers, respectively. None of the 592 cows screened in the present study had a homozygous mutant genotype. There was a statistically significant difference of the carrier rates between 2009 and 2018 ($P = 0.00257$).

Serum biochemical data for MPT

The results of serum biochemical analyses performed for 58 homozygous wild-type and four heterozygous carrier cows are shown in Table 1. The values are compared with the respective reference ranges reported in JB cattle (Watanabe et al., 2013a, Watanabe et al., 2013b; Watanabe et al., 2014; Shinya et al., 2020), which were measured under the same conditions as those used in the present study, and were partially updated for the purposes of this study. We detected no significant differences between the two genotypes with respect to any of the measured parameters. In almost all cases, the parameter values obtained for both groups were within or very close to the reference ranges, with the exceptions of TG, T-Cho and Glu concentrations in the carrier group. The mean TG concentration in the carrier group was

found to be considerably higher than the reference range, which can be attributed to the very high concentration (915.6 mg/*dl*) measured in one of the carrier cows. Compared with the respective reference ranges, this carrier cow also had notably high concentrations of T-Cho (249.3 mg/*dl*) and FFA (453.1 mmol/*l*) and a low concentration of Glu (36.5 mg/*l*). In addition, the Glu concentrations in the four carrier cows were all lower than the reference range.

Reproductive and developmental status

The data on reproductive and developmental performance are shown in Table 2. We found that all data relating to calves were very similar between the wild-type and carrier cows, whereas data relating to the treatment of cows indicated that the frequency and cost of treatments were slightly higher in the carrier cows than in the wild-type cows, although differences between the two groups were not significant.

1.5. DISCUSSION

To date, a number of genetic disorders have been reported in JB cattle, most of which, along with their causative mutations, are listed on the Online Mendelian Inheritance in Animals website (OMIA; https://omia.org/home/) as follows: Chediak-Higashi syndrome (OMIA 000185), factor XI deficiency (OMIA 000363), anhidrotic ectodermal dysplasia (OMIA 000543), Marfan syndrome (OMIA 000628), multiple ocular defects (OMIA 000733), renal dysplasia or claudin 16 deficiency (OMIA 001135), spherocytosis or band 3 deficiency (OMIA 001228), forelimb-girdle muscular anomaly (OMIA 001442), perinatal weak calf syndrome or IARS disorder (OMIA 001817), factor XIII deficiency (OMIA 001818), xanthinuria type II (OMIA 001819), hydrallantois or Bartter syndrome type 1 (OMIA 002053) and abortion or embryonic lethality (OMIA 002083), etc. In addition, there are several genetic disorders for which no causative mutations have been identified, including certain types of lysosomal storage disease (OMIA 000616) (Mikami et al., 2006; Masoudi et al., 2009), and maple syrup urine disease (OMIA 000627) (Kato et al., 2005). Collectively, these genetic disorders are assumed to have a substantial economic impact on the JB cattle industry; however, the details have yet to be evaluated.

In Japan, genetic testing for JB cattle is currently available for Chediak-Higashi syndrome, multiple ocular defects, claudin 16 deficiency, band 3 deficiency, forelimb-girdle muscular anomaly, IARS disorder, factor XIII deficiency, xanthinuria type II, and Bartter syndrome type

1, which are designated as defective genetic traits by the Ministry of Agriculture, Forestry and Fisheries of Japan since 2014 (Watanabe, 2017) and introduced by the Livestock Improvement Association of Japan (http://liaj.lin.gr.jp). These tests were applied to determine the genotypes of JB bulls based on semen samples stored for artificial insemination, and probably contribute to the prevention of these defective genetic traits in JB cattle. Among these, IARS disorder is recognized as having some of the most pronounced effects to the JB cattle industry, as it is one of the major causes of weak calf syndrome (Hirano et al., 2013; Hirano et al., 2016; Hirano et al., 2017), which for a long time has been a major factor contributing to perinatal mortality in JB calves (Ogata et al., 1999). Accordingly, in the present study, we specifically sought to investigate the prevalence and characteristics of IARS disorder in the JB cow population using a combination of our RT-PCR based genotyping assay and the MPT.

Genotyping using this RT-PCR assay revealed that the carrier rate for IARS disorder was 6.9% in 2009 and 1.5% in 2018. Kimura (2016) and Watanabe (2017) described that the mutant frequency of IARS ranged from 0.04 to 0.07 in JB beef cattle born from 2009 to 2012 surveyed in Japanese slaughterhouses, which is similar to the mutant frequency (0.035) in JB cows surveyed in 2009 in the present study. Compared to these data, the mutant frequency (0.0076) surveyed in 2018 in the present study seems to be very low. Furthermore, there was a statistically significant difference ($P<0.005$) between the carrier rates surveyed in 2009 and 2018 in the present study. There are several possible causes for the decrease in the carrier rate

from 2009 to 2018. These causes include the difference of farms (the different choice of semen), the change of popular regional bulls and the preferential exclusion of cows with producing weak calves at Kagoshima prefecture for nine years. The reproductive performance and resistance to diseases might be low in some carrier cows resulting in the preferential exclusion of such carriers, but this issue has not been statistically demonstrated in the present study due to a low number of carrier cows examined. Further studies are required to clarify this issue.

In general, it is possible that the limiting number of bulls could have resulted in an accidental decrease or increase in certain genetic traits, but a reduction of the genetic diversity in the regional population. The reduced genetic diversity may produce genetic disorders through inbreeding. Therefore, regional and/or nationwide reproductive control is required in order to maintain the appropriate genetic diversity by the prevention of extreme inbreeding and the avoidance of repeated use of very few bulls. However, given that the preventive approach of IARS has yet to be comprehensively adopted and recent carrier rate is considered sufficiently high, there remains a risk of producing cows having the mutation. By genotyping both JB cows and bull semen, the RT-PCR assay used in this study could make a useful contribution to the control and prevention of IARS disorder.

In this study, we compared the clinical and reproductive data obtained for 58 homozygous wild-type and four heterozygous carrier cows, which were derived from the most homogenous herd raised on the same cattle farm. Our results revealed no statistically significant differences

between the two genotypes with respect to any of the assessed MPT parameters, and with the exception of the concentrations of TG, T-Cho and Glu in the carrier group, almost all parameters in both groups were within or very close to the respective reference ranges (Table 1). One carrier cow was found to have notably high concentrations of TG, FFA and T-Cho and a low concentration of Glu, thereby indicating retarded lipid and energy metabolism in this cow. Indeed, retarded metabolism may be a common feature in carrier cows, given that we found that the Glu concentration of all carrier cows was below the reference range. In addition, among the reproductive and developing performance data (Table 2), we found that the frequency and cost of treatments were slightly higher for carrier cows than for wild-type cows, although differences between the two groups were not statistically significant. Hypothetically, carrier cows might be more susceptible to certain diseases and consequently require more treatment than wild-type cows due to the retarded metabolism; however, further studies are required to clarify this issue. Nevertheless, despite these deficiencies, our data indicate that the carrier cows have necessary IARS activity to maintain minimal health and reproductive potential that keep those data and performance in the reference range.

In a previous *in vitro* study in which cultured mouse cells were transfected with the bovine thymus cDNA from wild-type and affected animals, it was found that the aminoacylation activity of the mutant IARS protein decreases to 38% that of the wild-type protein (Hirano et al., 2013). This residual activity appears relatively high for an autosomal recessive trait. This

may explain, at least partially, why the carrier cows can maintain minimal health and reproductive potential. Pathogenic variations in genes encoding ARSs, including IARS, are increasingly being identified in association with human diseases, and the clinical features of ARS deficiencies appear very diverse and unpredictable (Fuchs et al., 2019). The same is true of livestock and particularly cattle populations. If there are certain underlining ARS mutations that are more deleterious than the *IARS* mutation, even carrier status may affect clinical and/or reproductive performances. MPT, used in conjunction with reproductive and developmental analyses, has the potential to detect such cows and/or entire cattle populations.

Table 1. Results of serum biochemical analyses in homozygous wild-type and heterozygous carrier cows for the *IARS* mutation

Analyte	Wild-type cows (n=58)	Carrier cows (n=4)	Reference range
TP (g/*dl*)	7.6 ± 0.4	8.0 ± 0.4	6.6–8.1
Alb (mg/*dl*)	3.4 ± 0.2	3.3 ± 0.2	3.0–3.8
A/G ratio	0.83 ± 0.14	0.72 ± 0.13	0.8–1.3
Glu (mg/*dl*)	42.2 ± 8.9	38.8 ± 2.4	44.6–67.0
BUN (mg/*dl*)	8.4 ± 1.5	7.5 ± 1.1	5.0–16.6
TG (mg/*dl*)	39.8 ± 84.2	252.2 ± 383.0	8.5–43.4
T-Cho (mg/*dl*)	136.4 ± 37.5	176.2 ± 42.7	76.7–141.7
FFA (mmol/*l*)	284.7 ± 186.8	340.9 ± 121.7	28.9–354.2
3-HB (μmol/*l*)	252.3 ± 121.2	224.3 ± 56.8	110.0–545.0
Ca (mg/*dl*)	8.8 ± 0.4	9.1 ± 0.3	8.8–10.4
iP (mg/*dl*)	5.2 ± 0.9	5.2 ± 0.4	4.2–6.7
Mg (mg/*dl*)	2.1 ± 0.2	1.9 ± 0.1	1.5–2.2
AST (U/*l*)	53.7 ± 8.5	62.6 ± 6.9	44.0–76.8
GGT (U/*l*)	18.9 ± 4.5	17.8 ± 7.2	11.3–21.6

The results obtained for each parameter are expressed as the mean ± standard deviation. There was no significant difference between the two groups. TP, total protein; Alb, albumin; A/G, albumin to globulin; Glu, glucose; BUN, blood urea nitrogen; TG, triglyceride; T-Cho, total cholesterol; FAA, free fatty acid; 3-HB, 3-hydroxybutyrate; Ca, calcium; iP, inorganic phosphorus; Mg, magnesium; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase.

Table 2. Comparison of the reproductive and developmental status of homozygous wild-type and heterozygous carrier cows for the *IARS* mutation

Performance	Wild-type cows (n = 58)	Carrier cows (n = 4)
Calf data:		
Number of live births	8.8 ± 0.7	9.0 ± 0.7
Birth weight of male calf (g)	29,730 ± 303	29,538 ± 322
Daily weight gain after castration (g/calf)	1.1 ± 0.1	1.1 ± 0.2
Birth weight of female calf (g)	26,084 ± 298	26,529 ± 25
Daily weight gain of female calf (g/calf)	0.9 ± 0.1	0.9 ± 0.1
Cow data:		
Frequency of treatment (/year/cow)	2.6 ± 1.8	3.3 ± 1.6
Treatment cost (Japanese yen/year/cow)	841 ± 620	1,066 ± 605

The results obtained for each performance parameters are expressed as the mean ± standard deviation. There were no significant differences between the two groups with respect to any of the assessed parameters.

Table 3. Primers and probes used in the real time (RT)-PCR assay and Sanger sequencing for bovine isoleucyl-tRNA synthetase (IARS) disorder

Primer/probe	Sequence 5′ to 3′ (mer)	Reporter (5′)	Quencher (3′)	Tm (°C)	Concentration (nM)
RT-PCR:					
Forward primer	GCAGGGACAATTAAAGATATAGTTACA AGATATGC (35)	NA	NA	59.7	450
Reverse primer	CCATGACAATCCCAGCCAAATCTT (24)	NA	NA	55.7	450
Probe for wild-type allele	TGGGTTTCACGTTGACAG (18)	VIC	NFQ	48.1	100
Probe for mutant allele	TGGGTTTCAC<u>C</u>TTGACAG (18)	FAM	NFQ	48.1	100
Sanger sequencing:					
Forward primer	CTACTGTTAGAGTTGCGGTC (20)	NA	NA	56.3	NA
Reverse primer	ACATCCCTGCCCTATGACAT (20)	NA	NA	56.3	NA

Tm = melting temperature calculated using OligoAnalizer 3.1 (https://sg.idtdna.com/calc/analyzer); NA = not applicable; VIC = 6-carboxyrhodamine; FAM = 6-carboxyfluorescein; NFQ = non-fluorescent quencher. The underlined letter in the sequence of the probe for the mutant allele indicates the corresponding guanine to a cytosine mutation (c.235G>C) in the bovine *IARS* gene.

CHAPTER 2

Frequency of an X-linked maternal variant of the bovine *FOXP3* gene associated with infertility in cattle: A comparative analysis of Japanese Black, Holstein Friesian, Korean Hanwoo, and Indonesian Madura cows

The above-titled work originally appeared in "*Animals* (Islam et al., 2022)" as: **Frequency of an X-Linked Maternal Variant of the Bovine *FOXP3* Gene Associated with Infertility in Different Cattle Breeds: A Pilot Study** authored by:

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2.1. ABSTRACT

Immune adaptation plays an essential role in determining pregnancy, which has been shown to be dependent on sufficient immunological tolerance mediated by FOXP3+ regulatory T cells. Recently, an X-linked maternal single-nucleotide polymorphism (SNP), located 2175 base pairs upstream of the start codon in the bovine *FOXP3* gene (NC_037357.1: g.87298881A>G, rs135720414), was identified in Japanese Black (JB: Bos taurus) cows in association with recurrent infertility. However, with the exception of JB cows, the frequency of this SNP has yet to be studied in other cow populations. In this study, we thus aimed to evaluate the frequency of this SNP in different cow breeds. Between 2018 and 2021, a total of 809 DNA samples were obtained from 581 JB, 73 Holstein Friesian (HF: *B. taurus*), 125 Korean Hanwoo (KH: *B. taurus coreanae*), and 30 Indonesian Madura (IM: a crossbreed between *B. indicus* and *B. javanicus*) cows, which were genotyped using a TaqMan probe-based real-time polymerase chain reaction assay designed in this study. The frequency of the G allele was found to be relatively high in local IM (0.700), moderate in dairy HF (0.466), and low in beef JB (0.250) and KH (0.112) cows, with differences in the frequencies be-tween each group being shown to be statistically significant (P<0.005) using Fisher's exact test. The results obtained in this study indicate that the G allele frequencies of the identified the SNP differ markedly in different breeds of taurine and indicine cattle. Given these findings, it would thus be important to evaluate the relationships between high frequencies of the G allele and

infertility in different breeds.

2.2. INTRODUCTION

Reproductive failure and infertility in cattle are of major concern in the dairy and beef industries worldwide. They are associated with the complex interactions among genetic, physiological, environmental, and managerial factors (Walsh et al., 2011). These factors contribute to problems with conception, inter-calving period, and delivery of healthy calves; postpartum complications, reduced milk yield; and assortative mating (Walsh et al., 2011; Deka et al., 2021). The fertility in lactating dairy cows is currently declining worldwide, with repeat breeding being identified as one of the most important reproductive problems affecting fertility (Yaginuma et al., 2019).

Maternal factors known to contribute to infertility include age, oocyte defects, endocrine dysfunction, nutritional and hormonal abnormalities, genital tract infections, and genetic alterations, although it is often difficult to identify the predominant cause when different precipitating factors coexist (Walsh et al., 2011). In recent years, the roles of maternal immune regulatory cells and their regulatory genes that may also contribute to infertility have gained increasing attention in both human and animal studies. In particular, the development, differentiation, and immune suppressive functions of regulatory T (Treg) cells have been a prime focus of numerous studies following the identification of *FOXP3*, a master gene that encodes a transcription factor regulating the development and function of these Treg cells (Li et al., 2015). Recent studies in human medicine have also demonstrated the vital role played in

maternal-fetal immunity in maintaining a normal pregnancy, which is associated with the induction of different immunocompetent cells. For example, dysfunctional CD4+ CD25+ Treg cells have been found to be linked to implantation failure (Li et al., 2015; Tsuda et al., 2019).

In 2001, a mutation in the *FOXP3* gene of scurfy mice was identified as a new marker for the suppressive effect of Treg cells (Hori et al., 2003), and since then, several mutations in this gene have been identified in human patients, particularly in males suffering from immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome (Bennet et al., 2001), as well as autoimmune diseases and preeclampsia (Chen et al., 2013). The effect of these mutations has been proposed to negatively affect the differentiation of maternal Treg cells, which could in turn promote fetus-specific effector T-cell activation and subsequent infertility (Arishima et al., 2017). Numerous studies have also detected an association between unexplained infertility or pregnancy complications and reduced endometrial *FOXP3* mRNA expression in women (Jasper et al., 2006), and the findings of a recent human study have also indicated that a mutation in the *FOXP3* promoter region is associated with recurrent spontaneous abortions in Chinese Han populations (Fan et al., 2018).

Contrastingly, despite the association between *FOXP3* gene variants and reproductive pathology, comparatively few studies have examined *FOXP3* gene related defects in veterinary medicine. In a recent genome wide association study (GWAS), however, an X-linked maternal single-nucleotide polymorphism (SNP), located 2175 base pairs upstream of the start codon in

the bovine *FOXP3* gene (NC_037357.1: g.87298881A>G, rs135720414), was identified in Japanese Black (JB: *Bos taurus*) cows in association with recurrent infertility (Arishima et al., 2017). Reporter assays indicated that this SNP G allele was associated with reduced levels of *FOXP3* transcription, which may be a maternal immunogenic factor underlying the identified association between recurrent infertility in repeat breeding cows and higher frequencies of the G allele (Arishima et al., 2017). Consequently, it is reasonable to speculate that this SNP could serve as a valuable target in efforts to enhance the fertility of cow herds.

Anti-Müllerian hormone (AMH) is an important hormone especially in female reproductive organs and is currently used as the best biomarker for a multitude of uses in reproductive medicine (Bedenk et al., 2020). Recently, in veterinary medicine, it is reported that the low AMH concentration in perinatal period of cows are associated with postpartum reproductive problems due to various maternal factors (Okawa et al., 2021). In addition, serum amyloid A (SAA) concentration is currently used as a good marker for inflammatory conditions in dairy cows, and the changes of SAA concentration are thought to be important for determining the prognosis of breeding cattle and improving the productivity and fertility (Shinya et al., 2022). In veterinary medicine, the relationship between these two biomarkers and the genotypes of the bovine *FOXP3* gene has yet to be studied in any breeds of cows.

To the best of our knowledge, with the exception of JB cows, the SNP has yet to be surveyed in populations of other bovine breeds. Accordingly, in this study, we specifically

sought to analyze the frequencies of this SNP in JB, Holstein Friesian (HF: *B. taurus*), Korean Hanwoo (KH: *B. taurus coreanae*), and Indonesian Madura (IM: a crossbreed between *B. indicus* and B. javanicus) cow populations. Besides, we also provide the first understanding of how AMH and SAA concentrations are associated with the three genotypes of the *FOXP3* gene mutation in HF heifers and parous cows.

2.3. MATERIALS AND METHODS

The experiments conducted in this study were performed in accordance with the guidelines regulating animal use and ethics at Kagoshima University (no. VM15041; approval date: 29 September 2015) and Yamaguchi University, Japan (no. 40, 1995; approval date: 27 March 2017), and oral informed consent was obtained from cooperating farmers.

Sample Collection and DNA Extraction

From 2018 to 2021, blood samples were collected from 809 clinically healthy cows (female): 581 JB, 73 HF, 125 KH, and 30 IM cows. The JB cows were born and raised on several private JB cattle farms in Kagoshima and Soo cities, Kagoshima Prefecture, Japan. The HF cows were derived from several commercial dairy herds in Fukuoka Prefecture, Japan. The KH cows were born and raised on several farms near Taegu City in Korea, and the IM cows were born and raised on Madura Island, Indonesia. The blood samples (no more than 1 mL) were obtained by jugular or caudal venipuncture and were spotted onto Flinders Technology Associates filter papers (FTA card; Whatman International Ltd., Piscataway, NJ, USA) and stored in a refrigerator (4 °C) until use for the extraction of DNA. DNA was extracted from discs punched out of the blood impregnated FTA cards following appropriate treatment, as previously described (Mizukami et al., 2011).

Genotyping of the SNP

The primers and TaqMan minor groove binder (MGB) probes used for the real-time polymerase chain reaction (RT-PCR) assays (the sequence of which are listed in Table 5) were designed based on the sequence of bovine *FOXP3* (NCBI Reference Sequence NC_037357.1). These primers and probes, each of which was linked to a fluorescent reporter dye (6-carboxyrhodamine or 6-carboxyfluorescein) at the 5′-end and a non-fluorescent quencher dye at the 3′-end, were synthesized by a commercial company (Applied Biosystems, Foster City, CA, USA). RT-PCR amplifications were carried out in a final volume of 5 μL consisting of 2× PCR master mix (TaqMan GTXpress Master Mix; Applied Biosystems), 80× genotyping assay mix (TaqMan SNP Genotyping Assays; Applied Biosystems) containing the specific primers, TaqMan MGB probes, and template DNA. A negative control containing nuclease free water instead of template DNA was included in each run. The cycling conditions consisted of 20 s at 95 °C, followed by 50 cycles of 3 s at 95°C and 20 s at 60 °C, with a subsequent holding stage at 25 °C for 30 s. The data obtained were analyzed using StepOne version 2.3 (Applied Biosystems). Several DNA samples with three different genotypes (A/A, A/G, and G/G) from four different bovine breeds were used to validate the genotyping assay, following genotype confirmation based on Sanger sequencing (Kazusa Genome Technologies Ltd., Kisarazu, Japan). The sequence around the SNP (NC_037357.1: g.87298881A>G, rs135720414) was confirmed based on the publicly available bovine genome sequence (ARS-UCD1.2).

Blood sample analysis for AMH and SAA

In the present study, blood samples from 69 HF parous cows with 1–8 parities (mean ± standard deviation = 3.17 ± 1.62) and 39 non-parous heifers were analyzed for AMH and SAA concentrations. AMH concentration was measured using a bovine AMH ELISA kit (AnshLabs, Webster, TX, USA), according to a method reported previously (Fushimi et al., 2019). Undiluted plasma (50 μL) was used for the assay, and the assay had a limited detection of 11 pg/mL and a coefficient of variation of 2.9% according to the manufacturer's instructions. SAA concentration was measured using an automated biochemical analyzer (Pentra C200; HORIBA ABX SAS, Montpellier, France) with a special SAA reagent for animal serum or plasma (VET-SAA 'Eiken' reagent; Eiken Chemical Co. Ltd., Tokyo, Japan) based on a previous report with similar measurement conditions (Otsuka et al., 2021). SAA concentration was calculated using a standard curve generated using a calibrator (VET-SAA calibrator set; Eiken Chemical Co. Ltd., Tokyo, Japan).

Statistical Analysis

Differences in allele frequencies between each group were statistically analyzed using Fisher's exact test, with *P* values of less than 0.05 considered to indicate a statistically significant difference. Differences in parities and days open between each group were

statistically analyzed using Wilcoxon rank sum test with *P* values of less than 0.05 considered to indicate a statistically significant difference. Correlation coefficient was analyzed using Pearson's product-moment correlation test, with *P* values of less than 0.05 considered to indicate a statistically significance. Statistical analyses were performed using R software.

2.4. RESULTS

In this study, we developed an RT-PCR assay using TaqMan MGB probes, which enables us to clearly identify all possible genotype combinations (A/A, A/G, and G/G). The genotyping results were found to be consistent with the Sanger sequencing results (Figure 1). The results of our survey of the four assessed cattle breeds are shown in Table 4. The A and G alleles were found to be present in all breeds examined in this study, with the frequency of the G allele being relatively high in IM (0.700), moderate in HF (0.466), and low in JB (0.250) and KH (0.112) cows. Collectively, the overall G allele frequency of the study cohort was 0.265. HF, JB, and KH cows were characterized by a predominant A allele at this locus, whereas in IM cows, the G allele was detected more frequently than the A allele. Moreover, differences in the allele frequencies between different groups were found to be statistically significant (P<0.005) using Fisher's exact test.

In HF parous cows, the number of A/A, A/G, and G/G genotypes were 23, 27, and 19, indicating that the G allele frequency was 0.471. Parities in A/A, A/G, and G/G genotypes were 3.35 ± 1.03, 3.48 ± 1.37, and 4.47 ± 1.98, respectively, and there was no significant difference between each group. Days open in A/A, A/G, and G/G genotypes were 447.7 ± 61.7, 426.9 ± 52.9, and 453.9 ± 53.3, respectively, and there was no significant difference between each group. AMH concentration (pg/mL) was found to be significantly (P < 0.05) higher in A/A genotype (626.0 ± 303.9) than in A/G (423.9 ± 272.5) and G/G genotypes (372.1 ± 252.4)

(Figure 2A). In SAA concentration (mg/L), there was no significant difference among the three genotypes: A/A (8.70 ± 10.82), A/G (7.90 ± 12.98), and G/G genotypes (18.67 ± 44.10) (Figure 2B). In HF non-parous heifers, there was no significant difference among the three genotypes in AMH concentration (pg/mL): A/A (585.1 ± 402.0), A/G (422.2 ± 265.2), and G/G genotypes (574.7 ± 507.2) (Figure 3A) and SAA concentration (mg/L): A/A (5.66 ± 10.02), A/G (10.36 ± 17.89), and G/G genotypes (3.70 ± 5.12) (Figure 3B). Furthermore, there was no significant correlation between AMH and SAA concentrations in both HF parous cows (Figure 4A) and non-parous heifers (data not shown). However, when three genotypes were analyzed separately, only A/A genotype in parous cows has negative correlation between AMH and SAA concentrations with particularly low probability ($P = 0.0528$) (Figure 4B).

2.4. DISCUSSION

In a previous GWAS analysis, Arishima et al (2017) identified an SNP (NC_037357.1: g.87298881A>G, rs135720414) in the upstream of the *FOXP3* gene of JB cattle. This SNP was associated with a reduction in *FOXP3* transcription, which in turn was linked to a reduction in the number of maternal Treg cells and led to infertility or repeat breeding (Arishima et al., 2017). Screening for this allele would ideally necessitate a simple reliable genotyping assay. In the present study, we accordingly designed a TaqMan MGB probe-based RT-PCR assay, the use of which provided clear-cut genotyping results (A/A, A/G, and G/G) for the non-risk type A and risk-type G alleles of the SNP. Moreover, by using FTA cards for sampling on cattle farms, we were able to eliminate the need for traditional multi-step DNA extraction and purification procedures, and this, combined with a relatively short amplification time (less than 1 h), facilitated the rapid genotyping and screening of the target SNP in less than 2 h, which is comparable with genotyping surveys performed for a range of bovine, canine, and feline genetic diseases (Mizukami et al., 2011; Kushida et al., 2015; Islam et al., 2021).

Our survey of different cattle breeds using the newly designed assay revealed that the risk of carrying the G allele was approximately 0.265, calculated from 458 A/A, 273 A/G, and 78 G/G genotypes detected in a population of 809 cows, and thus notably less likely than harboring the non-risk A allele (Table 4). The frequency of the G allele detected in JB cows in the present study (0.250) is similar to that (0.23) in the JB population screened by Arishima et al (2017).

Among the other assessed breeds, we found that the frequency of the G allele in KH cows (determined for the first time in this study) was significantly lower (0.112) than that in JB cows. Both JB and KH are beef cattle, which may have originally possessed the same maternal genetic factors associated with infertility and have unintentionally been selected with respect to the A allele. However, infertility and the problems associated with miscarriage/stillborns have yet to be sufficiently resolved in these beef breeds (Katagiri et al., 2013; Watanabe et al., 2013b; Watanabe et al., 2014; Kishida et al., 2015; Yi et al., 2022), thereby indicating that there remain other maternal factors responsible for these problems. Furthermore, the KH breed is believed to be a hybrid of taurine (B. taurus) and indicine (B. indicus) cattle (Rhee et al., 2001; Hur et al., 2008), and it is thus conceivable that the significant difference in G allele frequency between JB and KH cows may be attributable to the blood from indicine cattle.

Compared with that of the other three assessed breeds, the risk of carrying the G allele in HF dairy cows proved to be moderate (0.466). It would thus be particularly beneficial to focus on the SNP with regard to reducing the levels of infertility in these dairy cows. We are currently undertaking a follow up survey, in which we are examining the subsequent reproductive performance of each of the HF cow screened in the present study, with a view toward clarifying the associations between the genotype and fertility.

The IM breed of cattle is one of the Indonesian native bovine breeds developed by crossing zebu (B. indicus) and banteng (B. javanicus), the herds of which are predominantly

reared by small-scale farmers on Madura Island, East Java, not only for beef production, but also as working draught animals and for the purposes of racing and show (Popescu et al., 1988; Riszqina et al., 2008). In the present study, we established that compared with cows of the other three breeds, these cattle have a notably high likelihood of carrying the G allele (0.700), and we speculate that this high G allele frequency could be attributable to the genetic contribution of banteng cattle. However, this remains to be confirmed, given that frequencies of the G allele in these cattle have yet to be surveyed. Furthermore, it is speculated that the persistence of this detrimental allele in populations could be attributable to a lack of appropriate breeding management, as IM cattle are generally bred to maintain specific traits by natural mating (82.7%) with local IM breeding bulls (Yi et al., 2022). In this regard, there is also a high risk of inbreeding and thus the propagation of single mutant genes within the cattle herds, owing to the comparatively small number of IM bulls that are available for mating within the limited area in which they are reared (Riszqina and Smith, 2008). These conditions are conducive to considerable reductions in genetic variation, which may increase the prevalence of specific alleles such as the SNP G allele.

Among the major reproductive problems associated with IM cattle is repeat breeding (Nurgiartiningsih et al., 2016), which is conceivably linked to the high frequency of the G allele. If this is the case, the SNP would be a potentially valuable genetic marker. In order to maximize the utility of the SNP as a target in a range of cattle breeds, particularly the HF an IM breeds,

further studies will be necessary to evaluate the reproductive performance and biochemical parameters, such as metabolic profiles, among different breeds.

AMH is a glycoprotein secreted by ovarian granulosa cells primarily from pre-antral and early antral follicles of females, a reliable endocrine marker closely associated with the gonadotrophin responsive ovarian reserves and ovulation events (Monniaux et al., 2013; Mossa and Ireland, 2019). In this study, AMH concentration in HF parous cows with A/A genotype was significantly higher than that in cows with A/G and G/G, indicating that A/A cows have the higher ovarian potential than other two types of cows carrying the risk G allele. This observation suggests that the G allele of the *FOXP3* gene may negatively affect the excretion or production of AMH, resulting in the disadvantage in fertility. However, in this study, there was no significant difference in parities and days open between each group of the three genotypes in HF parous cows. Therefore, there may be additional factor(s) in combination of decreased concentration of AMH, which eventually result in infertility like repeat breeding.

The endometrium is the first line of defense against any organisms that ascend the female genital tract after parturition (Laura et al., 2015). The initial defense of the endometrium against microbes is also dependent on innate immune systems (Wira and Fahey, 2004; Nevers et al., 2011). Uterine inflammation due to clinical or subclinical endometritis, can also reduce reproductive function by suppressing hypothalamic gonadotropic secretion, follicular growth function, and ovulation, as well as reduced conception and pregnancy rates (Bell and Roberts,

2007). The AMH concentration can varies in individual cows throughout the reproductive life of animals, especially from the gestation to the postpartum period (Monniaux et al., 2013). The changes in AMH are related with inflammatory mediators, which may perturb the development of ovarian follicles (Sheldon et al., 2014). It is also shown that there is a negative relationship between AMH and SAA concentrations, indicating that certain infection and inflammation affect fertility of dairy cows (Okawa et al., 2021). In this study, there is no significant correlation between AMH and SAA concentration using all HF cows examined, but the probability ($P = 0.0528$) that is particular low and close to 0.05 was obtained in the correlation analysis in only wild-type A/A cows. In parous cows carrying the G allele, there was more cows with lower AMH regardless of SAA concentration. These observations suggest that cows carrying the G allele may be more susceptible to even minor types of infection and inflammation leading to the decrease in AMH excretion. The *FOXP3* gene plays a major role in controlling Treg cell dependent maternal immunity (Li et al., 2015; Tsuda et al., 2019), and therefore, the subsequent decrease of Treg cell due to the G allele of the *FOXP3* gene may be able to reduce maternal immunity and eventually allow the microbial invasion to the endometrium. These infectious and inflammatory conditions may be followed by the decrease in AMH excretion.

In HF heifers in this study, AMH and SAA concentrations did not differ among the three genotypes. Subclinical endometritis leading to subsequent postpartum infertility is more

common in parous cows than in heifers (Walker et al., 2015). Clinical or subclinical uterine disease can hamper ovarian function as well as AMH hormone production and thus affecting fertility which can be more frequent in high producing cows as the infection in uterus takes place more commonly right after calving that can persist long time and finally impact on postpartum fertility (Molina-Coto and Lucy, 2018). Heifers might have sufficient Treg-dependent immunity to protect the uterus against microbial infection resulting in relatively high AMH concentration and normal SAA concentration in this study. Heifers may be able to maintain a normal level of AMH and SAA because they are not experienced in calving and the genital tract remains almost closed to any infection (Okawa et al., 2021). The age and number of calving might be also important factors associated with fertility in combination with the genotypes of the bovine *FOXP3* gene.

Further studies will be required to demonstrate the relationship between the *FOXP3* gene SNP and infertility in different breeds of cows. In this regard, the genotyping assay developed in this study could make a notable contribution to surveying the bovine populations.

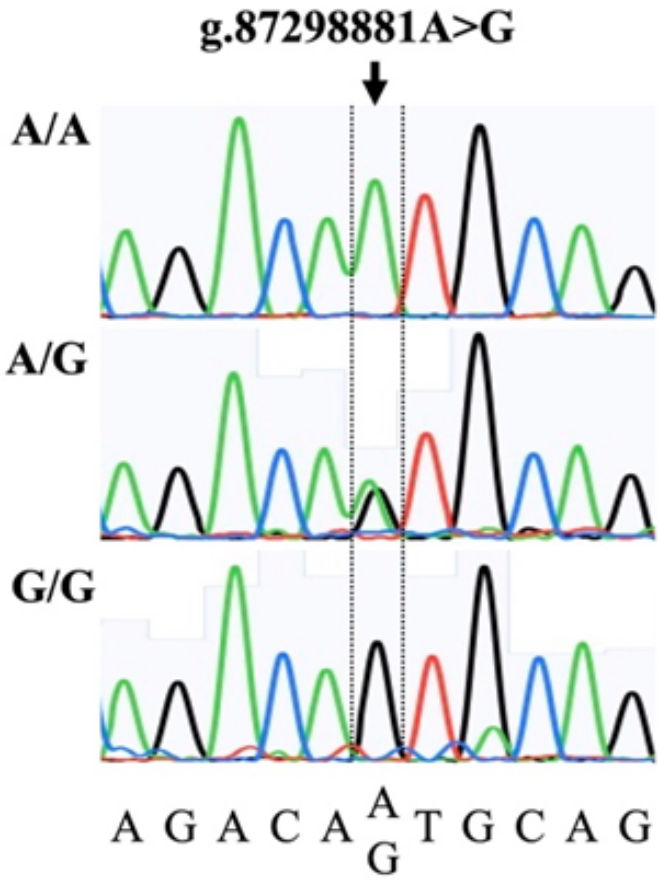

Figure 1. Representative Sanger sequencing electropherograms illustrating the A/A, A/G, and G/G genotypes associated with a single-nucleotide polymorphism (arrow; g.87298881A>G) in the upstream of the bovine *FOXP3* gene.

Table 4. The number of cattle genotyped and the frequencies for the bovine *FOXP3* single nucleotide polymorphism in different cattle breeds.

Cattle Breed	Number of Examined Cows	Number of A/A Allele (%)	Number of A/G Allele (%)	Number of G/G Allele (%)	G Allele Frequency
Japanese Black	581	333 (57.3)	205 (35.3)	43 (7.4)	0.250 *
Holstein Friesian	73	24 (32.9)	30 (41.1)	19 (26.0)	0.466 *
Korean Hanwoo	125	98 (78.4)	26 (20.8)	1 (0.8)	0.112 *
Indonesian Madura	30	3 (10.0)	12 (40. 0)	15 (50. 0)	0.700 *
Total	809	458 (56.6)	273 (33.7)	78 (9.6)	0.265

* Differences in frequencies between different groups were statistically significant ($P< 0.005$) using Fisher's exact test.

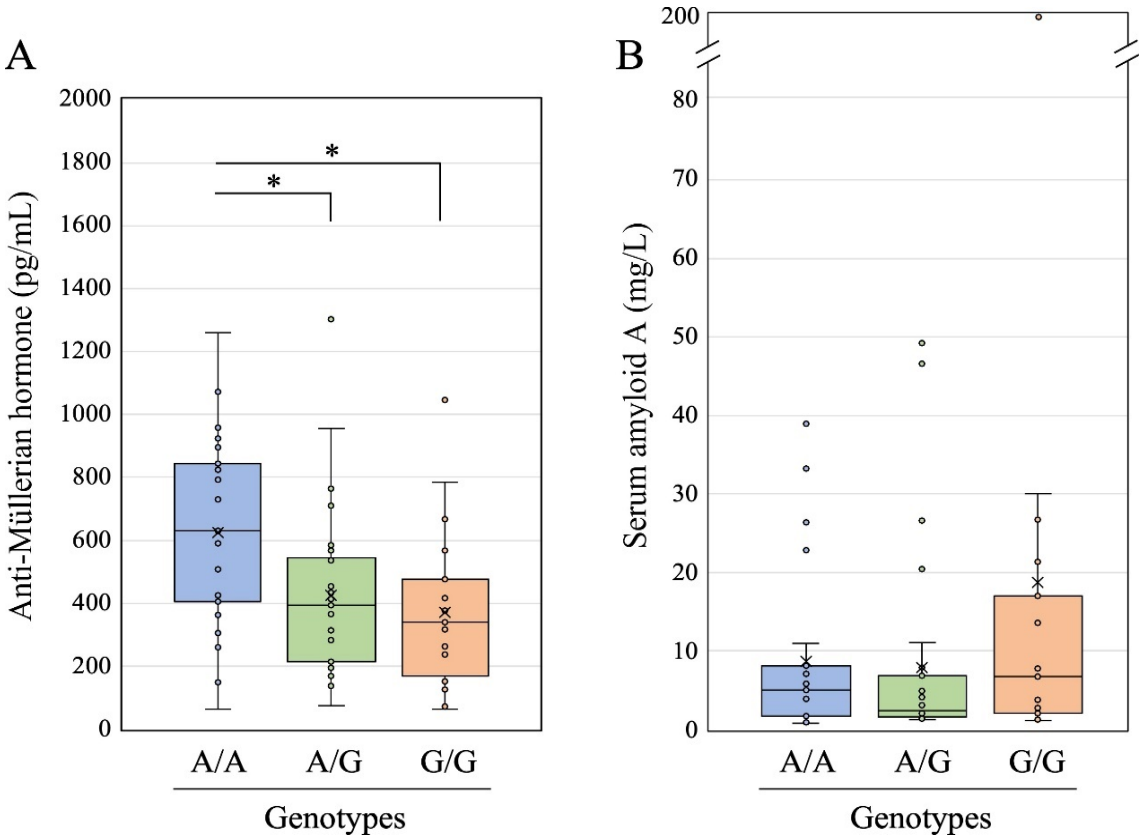

Figure 2. Anti-Müllerian hormone (AMH) and serum amylois A (SAA) concentrations in the three genotypes associated with a single nucleotide polymorphism in the *FOXP3* gene (g.87298881A>G) in HF parous cows. Boxplots with median (line within the box), interquartile range (box limits), and extremes (whiskers) of AMH (A) and SAA concentrations (B) in homozygous wild-type (A/A), heterozygous carrier (A/G), and homozygous mutant genotypes (G/G). * Differences between different groups were statistically significant ($P< 0.05$) using Wilcoxon rank sum test.

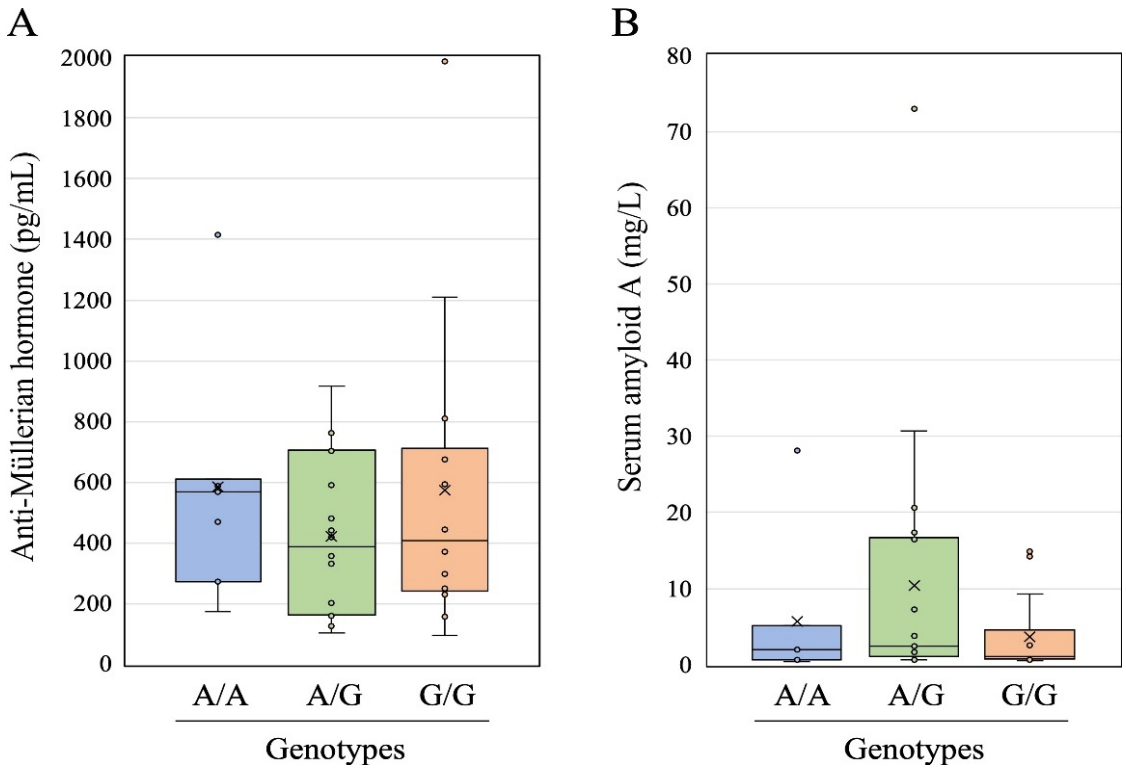

Figure 3. Anti-Müllerian hormone (AMH) and serum amylois A (SAA) concentrations in the three genotypes associated with a single nucleotide polymorphism in the *FOXP3* gene (g.87298881A>G) in HF non-parous heifers. Boxplots with median (line within the box), interquartile range (box limits), and extremes (whiskers) of AMH (A) and SAA concentrations (B) in homozygous wild-type (A/A), heterozygous carrier (A/G), and homozygous mutant genotypes (G/G).

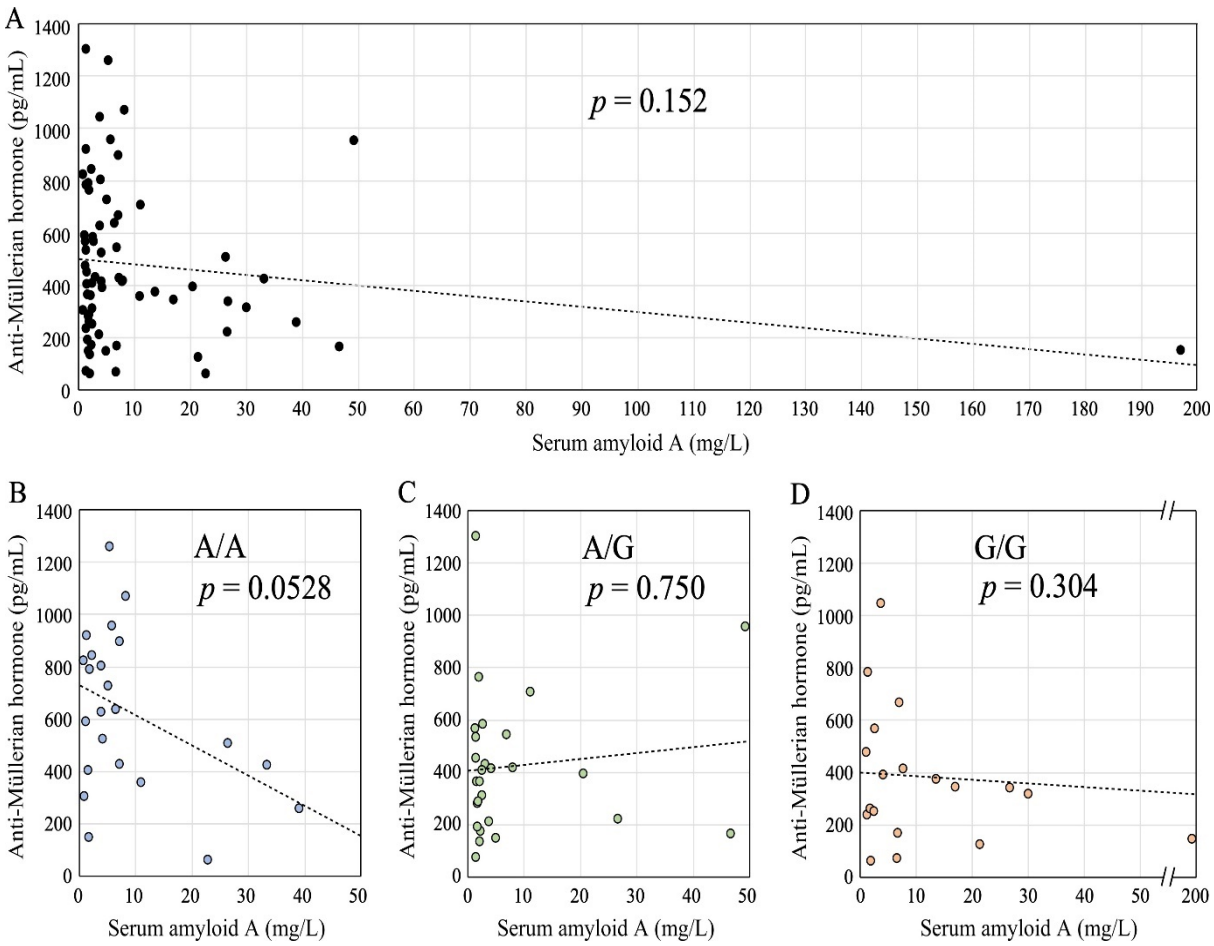

Figure 4. Correlation between Anti-Müllerian hormone (AMH) and serum amylois A (SAA) concentrations in all Holstein Friesian parous cows (A) and the cows with homozygous wild-type (A/A) (B), heterozygous carrier (A/G) (C), and homozygous mutant genotypes (G/G) (D) associated with a single nucleotide polymorphism in the *FOXP3* gene (g.87298881A>G). Probabilities (*p*) was calculated using Pearson's product-moment correlation test.

Table 5. Sequences of the primers and probes used in the real-time polymerase chain reaction (RT-PCR) assay and Sanger sequencing for a single-nucleotide polymorphism in the upstream of the bovine *FOXP3* gene.

Primer/probe	Sequence 5′ to 3′ (mer)	Reporter (5′)	Quencher (3′)	Tm (°C)	Concentration (nM)
	RT-PCR:				
Forward primer	CCATGTGGCTTCTGAGAAATAGTCA (25)	NA	NA	67.1	450
Reverse primer	TACCTGGAGGGCCAGACT (18)	NA	NA	62.3	450
Probe for the A allele	TCTTCCTGCATTGTCTG (17)	VIC	NFQ	50.0	100
Probe for the G allele	TCTTCCTGCACTGTCTG (17)	FAM	NFQ	52.0	100
	Sanger sequencing:				
Forward primer	AGGGCTCAGATGCAGAC (17)	NA	NA	54.0	NA
Reverse primer	GGATATGGTCTGTCTGGT (17)	NA	NA	54.3	NA

Tm, melting temperature calculated using Oligo Calculator (http://www.ngrl.co.jp/tools/0217oligocalc.htm) (accessed on 16 April 2022); NA, not applicable; VIC, 6-carboxyrhodamine; FAM, 6-carboxyfluorescein; NAQ, non-fluorescent quencher. The underlined letter in the sequence of the probe for the G allele indicates the corresponding adenine to a guanine transition (NC_037357.1: g.87298881A>G, rs135720414) in the upstream of the bovine *FOXP3* gene.

CONCLUSION

In this study (Chapter 1), genotyping of the JB population in Kagoshima prefecture revealed that there remains a risk of producing both carriers and affected cows for IARS gene mutation. There was no statistically significant difference in the MPT results or reproductive performance of carrier and wild-type cows, indicating that the carrier cows have necessary IARS activity to maintain minimal health and reproductive potential. By detecting anomalies such as underlying ARS deficiencies in cattle populations, these types of analyses could also be beneficially applied to facilitate maintenance of the equality of cow herds.

Furthermore, on the basis of the results obtained in this study (Chapter 2), it was established that current frequencies of the detrimental G allele of the SNP in the upstream of the bovine *FOXP3* gene are particularly high in local populations of IM cows compared with those of three other assessed breeds, among which, G allele frequencies are low in KH and JB cows, and moderate in HF dairy cows. Given these findings, it would thus be important to evaluate the relationships between high frequencies of the G allele and infertility in different breeds. Given that the moderate to high G allele frequencies in IM and HF cows are plausibly associated with the infertility of these breeds, it would be interesting to evaluate the relationship between the high frequency of the risk-type G allele and infertility. In this regard, the genotyping assay developed in this study could make a notable contribution to surveying the bovine populations.

In addition, on the basis of results regarding AMH and SAA, parous HF cows with wild-type A/A genotype had significantly higher AMH concentration than that in HF cows with other genotypes (G/A and A/A). Inflammatory condition indicated by an increased SAA concentration may potentially decrease AMH concentration particularly in G/A and A/A cows. These results suggest that the G allele may be associated with infertility in HF cows.

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