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

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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.

KEY WORDS: carrier rate, isoleucyl-tRNA synthetase (IARS) gene, Japanese Black cow, metabolic profile test, reproductive performance

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 [12, 14, 20]. 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 [1, 2, 12].

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 [3]. 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 [3–5]. 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 [4]. 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 [13] but also in beef cows [15–18]. 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 the same farm.

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, USA) 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 1. 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, USA). As DNA templates, we used DNA extracted from discs punched out of the blood-impregnated FTA cards, as previously described [10]. 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

Table 1. 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	GCAGGGACAATTAAAGATATAGTTACAAGATATGC (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*C*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; NAQ=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.

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 1.

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.

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 2. The values are compared with the respective reference ranges reported in JB cattle [15–18], 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

Table 2. Results of serum biochemical analyses in homozygous wild-type and heterozygous carrier cows for the isoleucyl-tRNA synthetase (*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 3. Comparison of the reproductive and developmental status of homozygous wild-type and heterozygous carrier cows for the isoleucyl-tRNA synthetase (*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.

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 3. 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.

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) [8, 9] and maple syrup urine disease (OMIA 000627) [6]. 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 [19] 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 [3–5], which for a long time has been a major factor contributing to perinatal mortality in JB calves [11]. 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 [7] and Watanabe [19] 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 nation-wide 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 2). 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 3), 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 [3]. 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 [2]. 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.

In conclusion, genotyping of the JB population revealed that the carrier rate was 6.9% in 2009 and 1.5% in 2018 at Kagoshima prefecture, although there remains a risk of producing both carriers and affected cows. We detected no statistically significant differences 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.

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