Effect of diagnosis and treatment of clinical endometritis based on vaginal discharge score and epidemiological research for risk factors associated with reproductive performance in Japanese dairy cows

（腟粘液スコアに基づく臨床型子宮内膜炎の診断、治療の効果と
繁殖成績に関与するリスク因子の疫学的解析）

The United Graduate School of Veterinary Science

Yamaguchi University

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We hereby recommend that the thesis prepared under supervision by Hiroaki Okawa, entitled “Effect of diagnosis and treatment of clinical endometritis based on vaginal discharge score and epidemiological research for risk factors associated with reproductive performance in Japanese dairy cows” should be accepted as fulfilling in part for the degree of Doctor of Philosophy.

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ABSTRACT

Reproductive efficiency and performance in dairy cows has been declining for 30 years, and optimal reproductive efficiency is necessary to guarantee profitability in the dairy industry. Clinical assessment of the reproductive status of early postpartum dairy cows, including the diagnosis and treatment of reproductive disorders, is essential to achieve optimal reproductive performance. In this regard, the reproductive performance of dairy cows is affected by various factors, such as reproductive disorders, postpartum disease, nutritional status, and environmental status. In the present study, we investigated the prevalence of clinical endometritis and to determine treatment efficiency in cows diagnosed with clinical endometritis using vaginal discharge score (VDS) grading system for vaginoscopy. In addition, we investigated the possible risk factors affecting reproductive performance using multivariate analysis.

In the first series of experiments, we evaluated the prevalence, effectiveness of diagnosis, and treatment based on VDS of clinical endometritis in dairy cows. To detect clinical endometritis and classify its severity, vaginoscopy was performed during 21 to 60 days postpartum in 164 Holstein cows, consisting of 229 lactations. Study groups were defined using the 4-point VDS scale, as follows: non-endometritis (VDS=0; no/clear mucus; NEM group; n=168); mild endometritis, no treatment (VDS=1; mucus containing flecks of white/off-white pus; NTR group; n=30); and severe endometritis, treated using PGF2α (VDS=2; discharge containing < 50% pus or VDS=3; discharge containing > 50% pus, and fluid or uterine horn asymmetry; TEM group; n=31). Cows treated using PGF2α

that did not recover (VDS≥1; n=5) received intrauterine procaine penicillin and streptomycin. The prevalence of clinical endometritis (VDS≥1) was 26.6%. The NTR group required significantly more artificial inseminations per pregnancy than the NEM and TEM groups (2.8±1.8 vs. 2.0±1.3 and 1.9±0.8, respectively; $P<0.05$). In the survival analysis, the proportion of non-pregnant cows was higher in the NTR group than in the NEM ($P=0.012$) and TEM ($P=0.076$) groups. In the TEM group, calving to the first artificial insemination interval tended to be higher in cows treated 41 to 60 days postpartum than in cows treated 29 to 40 days postpartum (97.2±27.1 vs. 74.4±19.7; $P=0.084$). The first study suggests that cows with a VDS of 1 may require treatment to recover fertility. Diagnosis and treatment of clinical endometritis based on a VDS grading system may improve dairy herd reproductive performance.

In the second experimental series, we aimed to determine possible risk factors affecting reproductive performance, especially time taken to establish pregnancy in dairy cows, using Cox's proportional hazard model. The data were collected from 154 Holstein Friesian cows (199 lactations). Cows diagnosed with a vaginal discharge score (VDS) of 1 or a calving abnormality showed significantly delayed pregnancy: hazard ratio (HR)=0.654 (95% confidence interval [CI]: 0.436–0.983; $P=0.041$) and HR=0.457 (95% CI: 0.270–0.774; $P=0.004$), respectively. The second study suggested that a VDS of 1 or calving abnormality may be risk factors increasing the number of days open and affecting reproductive performance in dairy cows.

GENERAL INTRODUCTION

Reproductive performance is a critical factor determining profitability in the dairy industry. However, reproductive efficiency and performance in dairy cows has been declining with the increase in milk production in Japan over the last 30 years [Nakada, 2006]. Conception and pregnancy rate have continued to decline, and the calving interval has continued to extend. To improve or maintain reproductive performance, regular clinical assessment of the reproductive status of postpartum dairy cows is essential and must include the diagnosis and treatment of reproductive disorders [Takagi *et al*., 2005].

Bovine endometritis is one of the most common postpartum disorders in dairy cows. It involves localized inflammation of the uterine lumen and is caused primarily by infection with the bacteria *Trueperella pyogenes* [Sheldon *et al*., 2009; Mido *et al*., 2015]. Most cows with uterine diseases exhibit anestrous with an extended luteal phase, and they can develop subfertility or infertility [Sheldon *et al*., 2009]. Clinical endometritis is defined by the occurrence of purulent or mucopurulent vaginal discharge detected more than 3 weeks postpartum [Sheldon *et al*., 2009]. The prevalence of clinical endometritis in dairy cows 15 to 60 days postpartum ranges from 23.6% to 42.6% [Gautam *et al*., 2009; Gautam *et al*., 2010; Leutert *et al*., 2012; Plöntzke *et al*., 2011].

In Japan, transrectal palpation of the genital organs is the most common technique used by veterinary practitioners to diagnose uterine and ovarian disease under field conditions, because it is a simple and quick cow-side technique [Otaki, 2011]. However,

the method is also insensitive and non-specific in the detection of endometritis [Gautam *et al*., 2010; McDougall *et al*., 2010].

In contrast, vaginoscopy is an efficient tool in the diagnosis of clinical endometritis; it allows clinicians to evaluate the presence and quality of vaginal discharge in the vaginal cavity and external cervical os [Gautam *et al*., 2009; Gautam *et al*., 2010; Leutert *et al*., 2012; Senosy *et al*., 2011]. It is a simple, inexpensive, and non-invasive method that is faster than alternatives such as cytobrush and biopsy. Vaginal discharge score (VDS) has been established as a 4-point scoring system to classify the nature of vaginal discharge and can be used to estimate reproductive prognosis [Gautam *et al*., 2009; Gautam *et al*., 2010; Sheldon *et al.*, 2009]. Despite this, these techniques are still used less often than rectal palpation by veterinary practitioners under field conditions [Otaki, 2011]. Therefore, it is necessary to evaluate the effectiveness of diagnosis and treatment based on VDS using vaginoscopy to popularize vaginoscopic examination in clinical fields relevant to Japan.

To treat clinical endometritis, efficient interventions are required, but veterinarians differ as to how the condition should be treated, and several different treatment approaches have been used, including hormone administration and intrauterine infusion of antibiotics or povidone-iodine [Mido *et al.*, 2015; Haimerl *et al*., 2014; Hirsbrunner *et al*., 2006; McDougall *et al*., 2013]. However, no valid criteria or optical timing and methods have been established [Dubuc *et al*., 2014; Hirsbrunner *et al*., 2006; Kasimanickam *et al*., 2005; Hirsbrunner *et al*., 2006; Mido *et al*., 2015; Tenhagen *et al*., 1999; Williams *et al*., 2005]. Antibiotic treatment has beneficial effects in cows with

clinical endometritis, but it is also associated with several problems, such as the emergence of antibiotic-resistant bacteria [Hamierl *et al*., 2014]. Moreover, in Japan, the commercial drug approved as an intrauterine antibiotic to treat endometritis requires a milk withdrawal period. Farmers prefer to avoid using antibiotics to treat high-producing dairy cows during the early postpartum period, so effective and safe treatments for endometritis without withdrawal periods are needed.

The administration of PGF2α requires no milk withdrawal period and is not associated with the emergence of resistant bacteria. Furthermore, strategic treatment of endometritis using PGF2α during the early postpartum period improves reproductive performance by accelerating uterine involution and luteolysis [Kasimanickam *et al*., 2005; Tenhagen *et al*., 1999]. However, veterinarians differ as to the benefits of such treatment, because its effects vary depending on whether the animal has a functional corpus luteum (CL) [LeBlanc *et al.*, 2002].

For this reason, Chapter 1 of the present study aimed to (1) determine the prevalence of clinical endometritis, identified at the postpartum fresh check (2) evaluate the effects of strategic PGF2α treatment on subsequent reproductive performance in Holstein cows using VDS grading system for vaginoscopy.

Reproductive performance can be influenced by conditions other than clinical endometritis, and many other related factors can contribute to postpartum diseases and conditions [Hayes *et al*., 2012; Zadeh *et al*., 2010; Sasaki *et al*., 2014]. Postpartum diseases in dairy cows negatively influence productivity and reproductive performance,

and the incidence of many common clinical diseases is closely associated with parturition [Goff *et al*. 1997]. In Japan, Goto *et al*. (2019) recently reported that cows with diseases occurring within 60 days of parturition demonstrated significantly lower 305-day energy-corrected milk (305ECM; -154 kg, 95% confidence interval [CI): -229 to -79] and risk of pregnancy (hazard ratio [HR): 0.85, 95% CI: 0.80–0.91], as well as higher risk of culling (HR: 1.36, 95% CI: 1.17–1.59] compared to cows with no diseases. Although several epidemiological studies have investigated the factors associated with productivity and fertility in dairy cows in Japan [Kino *et al*., 2019; Goto *et al*., 2019], none have focused on the influences of VDS findings during the early postpartum period. Among the many related factors that affect reproductive performance, including VDS of 1, researchers must identify those that have the greatest effect.

For this reason, Chapter 2 of the present study aimed to determine the possible risk factors affecting reproductive performance using multivariable analysis; the study focused on the effect of each factor on the time needed to establish successful pregnancy (number of days open).

Chapter 1

Effect of diagnosis and treatment of clinical endometritis based on vaginal discharge score grading system in postpartum Holstein cows

ABSTRACT

In this study, the prevalence, effectiveness of diagnosis, and treatment based on vaginal discharge score (VDS) of clinical endometritis in cattle were evaluated. To detect clinical endometritis and classify its severity, vaginoscopy was performed during 21 to 60 days postpartum in 164 Holstein cows consisting of 229 lactations. Groups were defined using the 4-point VDS scale. Study groups included the following: non-endometritis (VDS=0; no/clear mucus; NEM group; n=168); mild endometritis, no treatment (VDS=1; mucus containing flecks of white/off-white pus; NTR group; n=30); and severe endometritis, treated with PGF2α (VDS≥2; discharge containing <50% pus; and VDS=3; discharge containing >50% pus, and fluid or uterine horn asymmetry; TEM group; n=31). Cows treated with PGF2α that did not recover (VDS≥1, n=5) received intrauterine procaine penicillin and streptomycin. Prevalence of clinical endometritis (VDS≥1) was 26.6%. The NTR group required significantly more artificial inseminations per pregnancy than NEM and TEM groups (2.8 ± 1.8 vs 2.0 ± 1.3, 1.9 ± 0.8, P<0.05). In survival analysis, the proportion of non-pregnant cows was higher in the NTR group compared to the NEM (P=0.012) and TEM (P=0.076) groups. In the TEM group, calving to first artificial insemination interval tended to be higher in cows treated 41 to 60 days postpartum than cows treated 29 to 40 days postpartum (97.2 ± 27.1 vs 74.4 ± 19.7, P=0.084). Our study suggests that cows with VDS=1 may require treatment to recover fertility. Diagnosis and treatment of clinical endometritis based on a VDS grading system may improve dairy herd reproductive performance.

INTRODUCTION

Reproductive performance is a critical factor determining the profitability of dairy enterprises [Goto *et al.*, 2016]. Regular clinical assessment of the reproductive status of postpartum dairy cows, including the diagnosis and treatment of reproductive disorders, is essential to achieve optimal reproductive performance of dairy herds [Takagi *et al.*, 2005]. Postpartum uterine disease is a well-known cause of reduced reproductive efficiency of cattle [de Boer *et al.*, 2014; Gautam *et al.*, 2009; Gautam *et al.*, 2010; Hirsbrunner *et al.*, 2006; Plöntzke *et al.*, 2011; Sheldon *et al.*, 2006].

Endometritis occurs in the early postpartum period and is caused by a bacterial infection in the uterine lumen. Clinical endometritis is defined as purulent or mucopurulent uterine discharge detectable in the vagina during approximately 21 to 26 days postpartum [LeBlanc *et al.*, 2002; Sheldon *et al.,* 2009]. The prevalence of clinical endometritis in dairy cows 15 to 60 days postpartum ranges from 23.6 to 42.6% [Gautam *et al.*, 2009; Gautam *et al*., 2010; Leutert *et al.*, 2012; Plöntzke *et al.*, 2011]. Although different treatment methods have been utilized for endometritis, valid diagnostic criteria and optimal treatment timing have not been elucidated [Dubuc *et al.*, 2011; Hirsbrunner *et al.*, 2006; Kasimanickam *et al.*, 2005; Mcdougall *et al.*, 2013; Mido *et al.*, 2016; Tenhagen *et al.*, 1999; Williams *et al.*, 2005]. Rectal palpation of the genital tract is the most commonly used clinical method to detect uterine abnormalities due to its simplicity and rapid implementation in the field of veterinary practice [Otaki, 2011]. However, rectal palpation is one of the most insensitive and non-specific methods for detection of endometriosis [Gautam *et al.*, 2009; McDougall *et al.*, 2010]. Vaginoscopy is an efficient

diagnostic tool to diagnose clinical endometritis that is practical for use in field conditions [Gautam *et al.*, 2009; Gautam *et al.*, 2010; Leutert *et al.,* 2012; Senosy *et al.*, 2011]. It is simple, inexpensive, and can be rapidly performed compared to alternative methods such as cytobrush, biopsy, and ultrasound, but is less frequently used than rectal palpation [Otaki, 2011].

A vaginoscope, with the aid of a flashlight, is used to visually detect the presence and quality of vaginal discharge [Leutert *et al.*, 2012]. A 4-point scoring system, the vaginal discharge score (VDS), has been used to classify the nature of the vaginal discharge [Leutert *et al.*, 2012; Mido *et al.*, 2016; Sheldon *et al.*, 2009; Williams *et al.*, 2005]. Although the VDS grading system is well-known as a useful method to diagnose clinical endometritis and to estimate reproductive prognosis [Gautam *et al.*, 2009; Gautam *et al.*, 2010; Sheldon *et al*., 2009], information regarding the effectiveness of treatment for clinical endometritis using a VDS grading system under field conditions is lacking and controversial.

Gautam *et al.* [Gautam *et al*., 2009; Gautam *et al*., 2010] studied the prevalence of clinical endometritis and its impact on subsequent reproductive performance in Japanese dairy herds. However, they did not investigate the efficacy of clinical treatments based on the VDS. Moreover, according to the clinical guidelines of the Agricultural Disaster Compensation System of the Ministry of Agriculture, Forestry, and Fisheries of Japan, the initiation date of treatment for cows diagnosed with endometritis is limited to 40 days postpartum or later [Ministry of Agriculture, Forestry, and Fishers, 2014]. Supporting scientific evidence of this limitation is unclear, and the clinical guidelines of this reference have not been revised since 1999. This guideline might cause a delay in the optimal timing of treatment of cows with clinical endometritis. Hence, we needed to clarify whether

treatment for cows with endometritis before 40 days in milk (DIM) is effective or not in Japanese dairy herds.

This study was conducted in commercial dairy herds under field conditions. The objectives of this study were 1) to determine the prevalence of clinical endometritis, identified at the postpartum fresh check, and 2) to evaluate the effectiveness of strategic treatment based on a VDS grading system by vaginoscopy on subsequent reproductive performance in Holstein cows.

MATERIALS AND METHODS

Animals and management

The study was conducted using 164 Holstein Friesian cows (total 229 lactations). Cows calved from September 2013 to December 2015, a 27-month-period. The cows were located in four commercial dairy herds in the Fukuoka prefecture, Kyushu, the largest island in southern Japan, where ambient average temperature ranges from a low of 1°C during the winter to a high of 30°C during the summer. The size of the lactating herd varied from 20 to 50 cows. The herds were non-seasonal, milked twice daily, and average milk production varied between 8,100 and 10,900 kg/cow/year. Parity ranged from one to eight. Cows were managed in a free-stall barn, bedded with wood shavings (Herd A), or in a tie-stall barn with rubber mattresses (Herds B, C and D). In Herd A, the behavior of standing to be mounted was considered to be a sign of estrus, and cows were artificially inseminated 8 to 14 hr after standing estrus. In herds B, C and D, estrus detection was based on hyperemia and swelling of the vulva, mucus discharge, bellowing, and restlessness. Cows were inseminated 8 to 14 hr after observing these signs. The voluntary waiting period in each herd was set at 40 days postpartum. Cows were bred by artificial insemination (AI) performed by local technicians. Each herd was visited monthly for a clinical reproductive examination including treatment of reproductive disorders and pregnancy diagnosis. Pregnancy was diagnosed by transrectal palpation or ultrasonography 40 days after the previous insemination. For cows not inseminated within 90 days postpartum, or not pregnant at the time of pregnancy diagnosis, additional investigation and treatment were performed, such as a timed AI program using a combined injection of PGF2α and estradiol.

Clinical examination and treatment of endometritis

A monthly reproductive medical examination was performed from October 2013 to December 2015; it included vaginoscopy and the transrectal palpation of reproductive organs during the fresh check after parturition. In our study, the "fresh check" refers to the first examination of the genital tract after parturition, including the ovaries and uterus. The fresh check was conducted in all cows 21 to 60 days after parturition. First, transrectal palpation of the uterus and ovaries was performed to determine the size, location, and symmetry of the uterine horns and the presence of palpable ovarian structures. Next, the vaginoscopic examination was performed to detect endometritis and to score the appearance of the vaginal discharge. To perform vaginoscopy, the tail was held to one side by the farmer, and the vulva was washed with lukewarm water containing [mono, bis-(methylene trimethyl ammonium chloride)]-alkyl (C9-15) toluene (Pacoma®; 10% solution; Scientific Feed Laboratory Co., Ltd., Tokyo, Japan) at a 1:1,000 dilution and wiped with a clean paper towel. A stainless vaginal speculum (Fujihira Industry Co., Ltd., Tokyo, Japan; length: 270 mm, external diameter: 35 mm) was inserted into the vagina to the external cervical os. The cervix and vagina were visually examined for the presence and quality of discharge with the help of a flashlight as previously described [Leutert *et al.*, 2012]. The VDS was used to classify the discharge: 0 =no or clear mucus; 1 =mucus containing flecks of white or off-white purulent material; 2 =discharge containing less than 50% purulent material; and 3 =discharge containing more than 50% purulent material and a small amount of fluid or asymmetry of the uterine horns detected by transrectal palpation. Findings from rectal palpation and the VDS were recorded for each examination.

Cows classified with a VDS of 2 or above were treated with 25 mg of Dinoprost (PGF2α) by IM injection (Pronalgon®F; Zoetis Japan Co., Ltd., Tokyo, Japan), irrespective of transrectally palpable corpus luteum (CL) status. In contrast, because most cases of mild endometritis recover spontaneously [Gautam *et al.*, 2010], cows with a VDS of 1 or less received no further treatment or reassessment. Cows treated with PGF2α were reassessed using the same methods 27.8 ± 12.8 day after the initial treatment. A clinical cure was confirmed by no or clear mucus discharge (VDS of 0). Of the cows with treated with PGF2α, five were inseminated during a spontaneous estrus prior to re-assessment of VDS; these cows had no or clear mucus discharge confirmed by the farmer and AI technician and were defined as clinically cured. All cows that were not defined as clinically cured were treated with an intrauterine infusion of antibiotics containing an oil mixture of benzyl procaine penicillin (400,000 IU) and dihydrostreptomycin sulfate (400 mg) in 30 ml of ointment base (Foamingmycin for endometritis®; Riken Chikusankayaku Co., Ltd., Tokyo, Japan). No further clinical assessment or treatment was conducted after the second treatment. A flowchart of the diagnosis and treatment protocol is shown in Fig. 1.

Data collection and statistical analysis

Uterine conditions are dynamic during the postpartum period, and uterine involution in dairy cows is often completed by 28 to 40 days postpartum [de Boer *et al.*, 2014; Plöntzke *et al.*, 2011; Sheldon *et al.*, 1998]. To investigate the prevalence of endometritis after parturition, the VDS data were analyzed in three DIM groups: 21 to 28, 29 to 40 and 41 to 60 days. In addition, the VDS data were analyzed for the overall DIM range (21 to 60 days). The prevalence of various degrees of endometritis, based on the

VDS, was expressed as a percentage of total cows examined during the same period.

To determine the impact of a VDS-based treatment of endometritis on reproductive performance, the cows were divided into three groups: non-endometritis (NEM group, n=168; VDS=0); non-treated, mild endometritis (NTR group, n=30; VDS=1); and treated, severe endometritis (TEM group, n=31; VDS≥2). The mean days postpartum of the three groups (NEM, NTR and TEM) at the time of examination and treatment for clinical endometritis were 39.6 ± 9.1, 37.2 ± 9.4 and 36.1 ± 9.6, respectively; the difference among the three groups was not significant (P=0.84).

To evaluate the efficacy of treatment, data from the TEM group were further subdivided according to the presence or absence of a transrectally palpable CL and according to the timing of treatment (21 to 28, 29 to 40 and 41 to 60 DIM). Animals clinically cured after the initial treatment were also recorded in the TEM group. Of the 31 cows in the TEM group, two were culled before becoming pregnant. Reproductive performance data were compared among the three groups and included the following: calving to first AI (FAI) interval, successful conception after FAI, the number of AI required to become pregnant, and the number of days open. Reproductive performance data were collected until confirmation of pregnancy, culling, or death.

To evaluate the effects of reproductive management on dairy herds under farm conditions, the average monthly change in days open and the proportion of pregnant cows were analyzed.

The results are expressed as the mean ± standard error of the mean (SEM) or as a percentage. Statistical analyses were performed using Ekuseru-Toukei 2012 for Windows, version 1.11 (Social Survey Research Information Co., Ltd., Tokyo, Japan). The results for reproductive parameters of cows with or without a palpable CL in the TEM group

were evaluated using the Mann-Whitney nonparametric U-test. The results for reproductive parameters of the three groups (NEM, NTR and TEM) and cows classified according to timing of treatment (21 to 28, 29 to 40 and 41 to 60 DIM) in the TEM group were analyzed using one-way analyses of variance (ANOVA) and Tukey-Kramer's post hoc test. Pregnancy rates and the clinical cure rate were compared using the $\chi 2$ test or Fisher's exact probability test. The data on the proportion of cows from the three groups that did not become pregnant (survival rate) were visualized using Kaplan-Meier survival curves, and the differences were analyzed using a generalized Wilcoxon test. A P value <0.05 was considered statistically significant.

RESULTS

Prevalence of endometritis

The prevalence of endometritis based on the VDS grading system is shown in Table 1. The prevalence in each herd was A:32.4% (11/34), B: 27.1% (13/48), C: 24.5% (24/98) and D: 26.5% (13/49). The percent of cows with a VDS of 1 or more, 21 to 28, 29 to 40, and 41 to 60 days postpartum, was 41.9% (13/31), 25.5% (26/102), and 22.9% (22/96), respectively. The overall prevalence of endometritis within the 60-day postpartum period was 26.6% (61/229). Thirty-one cows with a VDS of 2 or more were treated with PGF2α.

Effect of treatment of clinical endometritis categorized using a VDS grading system on reproductive performance

Reproductive performance of cows in three groups is shown in Table 2. Although conception rates, days to FAI, and days open were not different among the groups, the NTR group required a significantly higher number of AI to become pregnant as compared to either the NEM or TEM groups (P=0.014 and P=0.032, respectively).

Survival curves of the three groups are shown in Fig. 2. Pregnancy rates within 150 days postpartum were 69.6% (117/168), 64.5% (20/31), and 50.0% (15/30) in the NEM, NTR and TEM groups, respectively. In the survival analysis, the proportion of non-pregnant cows in the NTR group was significantly higher as compared to the NEM groups (P=0.012), and also tended to be higher than the TEM group (P=0.076).

Table 3 shows the clinical cure rates and reproductive performance of cows with or without a palpable CL and according to the timing of the treatment. The proportion of clinically cured cows was 83.9% (26/31). There was no association among the proportion

of clinical cures, the presence or absence of a palpable CL, or the timing of treatment with PGF2α. The calving to FAI interval tended to be longer in cows treated with PGF2α at 41 to 60 days postpartum as compared to cows treated 29 to 40 days postpartum (P=0.084). There were no differences between groups in the status of the CL, timing of PGF2α, conception after FAI, number of inseminations, or number of days open.

The average monthly change in mean days open and the proportion of pregnant cows were not significantly different over time (Fig. 3). There was a similar variation in the proportion of pregnant cows per farm every 12 to 14 months. After the implementation of the VDS grading system at the fresh check, there was a tendency towards an increase in the proportion of pregnant cows and a decrease in number of days open.

DISCUSSION

The main objectives of the present study were to investigate the prevalence of clinical endometritis and to determine the efficiency of treatment of cows diagnosed with endometritis based on a VDS grading system for vaginoscopy under field conditions. We demonstrated that cows treated with PGF2α by clinical endometritis recovered and regained fertility more quickly; their recovery and fertility were comparable to cows without endometritis (VDS of 0). Furthermore, early treatment (29 to 40 days postpartum) may have had a positive effect in the management of dairy herds. In contrast, cows that did not receive treatment for mild endometritis (VDS of 1) showed lower reproductive efficiency.

Uterine infection within one week of parturition is identified in 40% of dairy cows. The infection rate decreases over time, and approximately 15 to 20% of cows develop clinical endometritis [de Boer *et al.*, 2014; Sheldon *et al.*, 2009]. In two field studies in Japanese dairy herds, Gautam *et al*. reported that the prevalence of clinical endometritis within 60 d postpartum was 32.6% (44/135) and 23.6% (104/441) [Gautam *et al,*, 2009; Gautam *et al.*, 2010]. In the present study, the prevalence of clinical endometritis 21 to 60 days postpartum was 26.6% (61/229), which is comparable to that reported previously. [Gautam *et al.*, 2009; Gautam *et al.*, 2010]. A previous study suggested that the presence of mild endometritis with a VDS of 1 may not have an adverse effect on reproductive performance, and that a small amount of vaginal discharge in the early postpartum period may be part of the physiologic self-cleaning process [Gautam *et al.*, 2010; Hirsbrunner *et al.*, 2006]. It is unclear if cows with mild endometritis should be treated [Gautam *et al.*, 2010; Leutert *et al.*, 2012; McDougall *et al*., 2013]. On the basis of previous reports

[Gautam *et al.*, 2010; Leutert *et al.*, 2012], we assumed that cows with mild endometritis had the ability to spontaneously recover. However, we observed that this group of cows had evidence of negative reproductive performance, such as an increase in the number of inseminations as compared to the other two groups. Thus, cows with a VDS of 1 may have had persistent mild endometritis, which impaired fertility. Our study found no difference in reproductive performance between the NEM and TEM groups. These data suggest that cows with mild endometritis require treatment to recover uterine condition and fertility, similar to cows with severe endometritis.

Vaginoscopy has been shown to have a relatively high specificity (87–96%) as compared to metricheck and ultrasound analyses (62–78%). The sensitivity of vaginoscopy is moderate (54–72%) [de Boer *et al*., 2014]; visual assessment of VDS using a flashlight in this study may cause the error of defining a VDS of 1 or above as a 0 [Leutert *et al.*, 2012]. However, we reassessed cows with severe endometritis alone in the TEM group, and did not reassess cows with mild endometritis in the NTR group. Gautam *et al*. reported that 10.6% (31/335) of cows with no endometritis on vaginoscopy during 15 to 60 days postpartum developed endometritis beyond 60 days postpartum [Gautam *et al*., 2010]. The cows with mild or no endometritis in this study might have shown severe endometritis if the second vaginoscopic examination was performed in the NEM, NTR group; hence, reassessment of uterine condition using vaginoscopy might be required in the cows with mild or no endometritis during 21 to 60 days postpartum. The definitive diagnostic method for endometritis, including subclinical disease, is a cytological assessment demonstrating the percentage of polymorphonuclear neutrophils (PMNs) at 35 to 40 days postpartum. This is because >5% PMNs generally indicates impaired reproductive performance [de Boer *et al*., 2014]. In addition, a decrease in

bacterial infection and the percentage of PMNs is reflected in a reduction of the VDS [Mido *et al*., 2016]. Although cytology is a very useful method for diagnosing endometritis, this method is less suitable for clinical practice because it is more costly and time consuming. Therefore, we must consider that some cows classified as having no or mild endometritis by vaginoscopy may in fact have had subclinical endometritis. Although previous reports determined that vaginoscopy is not a perfect diagnostic method for clinical endometritis, it may be better suited as a field diagnostic method in clinical practice [Gautam *et al.*, 200; Gautam *et al.*, 2010; Leutert *et al.*, 2012]. In clinical practice in the field, there are no criteria available to determine if clinical endometritis should be treated. The present study suggests that mild endometritis, with a VDS of 1 based on vaginoscopy, requires reassessment and treatment to recover uterine health and fertility.

In the present study, we administered PGF2α as an initial treatment for cows with clinical endometritis, irrespective of their CL status. The proportion of subjects with clinically cured endometritis was 83.9% (26/31). This is higher than the proportion reported by previous studies with cure rates of 77 and 81.1% [LeBlanc *et al.*, 2002; McDougall *et al.*, 2013]. We reassessed the VDS 29.1 ± 12.8 days after initial treatment, which is longer than the duration reported by a previous study (approximately 14 days after treatment) [Dubuc *et al*., 2011; LeBlanc *et al.*, 2002; McDougall *et al.*, 2013]. This difference may have influenced the clinical cure rate. At the time of reassessment, the cows that were not clinically cured were given a second treatment of intrauterine antibiotic infusion. Intrauterine infusion of antibiotics as the first treatment requires a 24-hr milk withdrawal period in Japan, and is associated with development of antimicrobial resistance [Haimerl *et al.*, 2014]. Conversely, the administration of PGF2α does not require a milk withdrawal period and is not associated with the emergence of resistant

bacteria. Strategic treatment with PGF2α during the early postpartum period efficiently treats endometritis and improves reproductive performance by accelerating uterine involution and luteolysis [Kasimanickam *et al.*, 2005; Tenhagen *et al.*, 1999]. However, Dubuc *et al*. reported that two treatments of PGF2α (administered 35 and 49 days postpartum) for endometritis diagnosed by cytological assessment did not improve reproductive performance [Dubuc *et al.*, 2011]. Thus, it might be possible that only the severe cases of endometritis, and not the mild cases, benefit from treatment.

In the present study, the status of a palpable CL and the timing of the treatment did not affect the proportion of clinical cures. A previous report suggested that treatment of endometritis with PGF2α is successful if the cow has a CL (with a high progesterone concentration in the milk) at the time of treatment [Senosy *et al.*, 2011]. LeBlanc *et al*. reported that cows without a palpable CL during 20 to 26 days postpartum should not be treated with PGF2α; rather, these cows should receive treatment for endometritis beyond 28 days postpartum [LeBlanc *et al.*, 2002]. In contrast, Hirsbrunner *et al*. reported that combined treatment of PGF2α and prostaglandin E during 21 to 35 days postpartum had a positive effect on the number of days to FAI for cows with endometritis but without a CL [Hirsbrunner *et al.*, 2006]. Thus, the efficacy of PGF2α treatment for cows with endometritis is unclear and controversial. In the present study, although we demonstrated that PGF2α treatment during 29 to 40 days postpartum tended to shorten the calving to FAI interval as compared to treatment during 41 to 60 days postpartum, we could not clarify whether the status of CL influenced the clinical cure rate and reproductive performance after PGF2α treatment. In addition, the second treatment of five cows with severe endometritis using intrauterine antibiotic infusion in the TEM group might have a positive influence on the reproductive performance. Ovarian status and postpartum days

in dairy cows are strongly associated and influence each other [Senosy *et al.*, 2011; Takagi *et al.*, 2005]; consequently, further studies using stratified analysis are needed to avoid confounding. Senosy *et al*. reported that diagnostic methods such as cytology, metricheck, and vaginoscopy were influenced by ovarian status and uterine conditions (involution, tonicity, flatus, and flaccidity) [Senosy *et al.*, 2011]. Therefore, the diagnosis and treatment of cows with endometritis should be conducted under field conditions based on ovarian status, uterine condition, and VDS. The routine use of ultrasonography may improve reproductive efficacy and may increase the accuracy of identifying a CL and uterine abnormalities [Takagi *et al.*, 2005].

To the best of our knowledge, this is the first study on long-term regular reproductive practices, including clinical diagnosis and treatment based on the VDS grading system using vaginoscopy, which is useful and suitable for routine reproductive practice, and has a beneficial effect on the reproductive performance of dairy cows under field conditions. Conversely, cows with mild endometritis that were not treated showed a negative reproductive progression. Since the cows with mild endometritis in this study were left untreated, further controlled studies are needed to confirm whether treatment of such cows improve their reproductive performances or not. To establish a gold standard for the diagnosis and treatment of endometritis in clinical field practices, further studies are needed that consider the costs, time, required skill, and invasiveness of the method, as well as ovarian and uterine conditions.

FIGURE LEGENDS

Fig. 1. Flow chart of clinical diagnosis and treatment based on the VDS grading system.

Fig. 2. Kaplan-Meier survival curves for the proportion of open cows in the three treatment groups. NEM group (VDS=0, no endometritis): n= 168; median days open = 112; 5.4% (n=9) censored. NTR group (VDS=1, non-treated, mild endometritis): n= 28; median days open = 148; 7.1% (n=2) censored. TEM group (VDS≥2, treated, severe endometritis): n= 29; median days open = 128; 6.9% (n=2) censored.

Fig. 3. Mean open period and proportion of pregnant cows in each month in four dairy farms. The arrow (↑) indicates the beginning of reproductive management at fresh check, including the vaginal discharge scoring system.

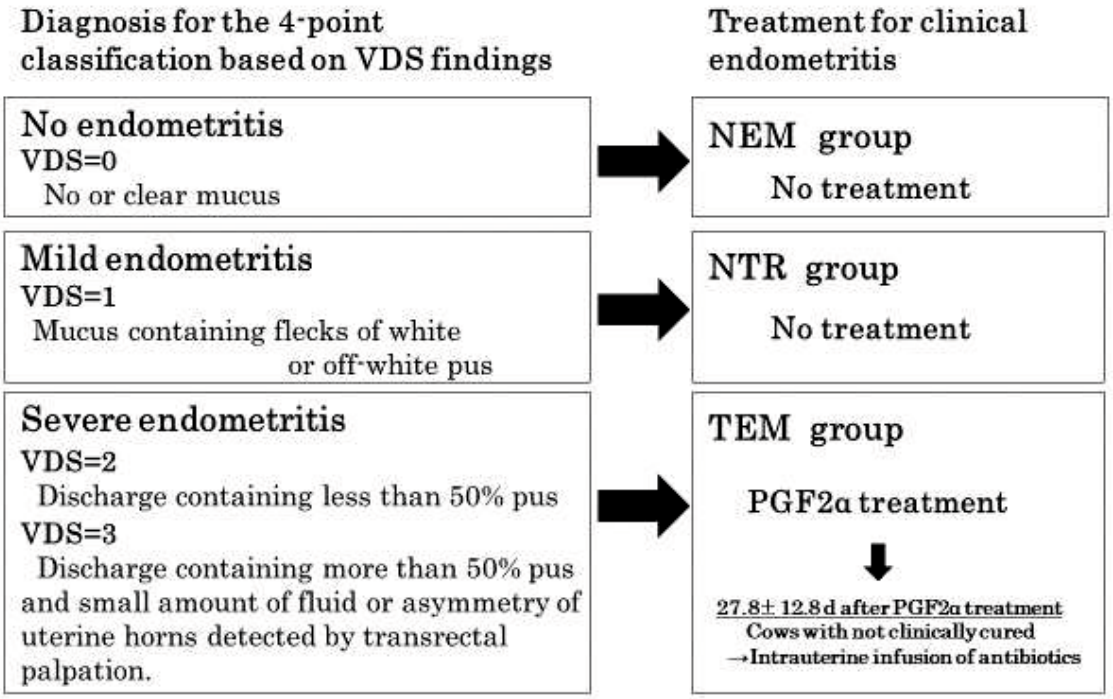
Diagnosis for the 4-point classification based on VDS findings
Treatment for clinical endometritis
No endometritis
VDS=0
No or clear mucus
NEM group
No treatment
Mild endometritis
VDS=1
Mucus containing flecks of white or off-white pus
NTR group
No treatment
Severe endometritis
VDS=2
Discharge containing less than 50% pus
VDS=3
Discharge containing more than 50% pus and small amount of fluid or asymmetry of uterine horns detected by transrectal palpation.
TEM group
PGF2α treatment
27.8± 12.8 d after PGF2α treatment
Cows with not clinically cured
→Intrauterine infusion of antibiotics

Fig.1

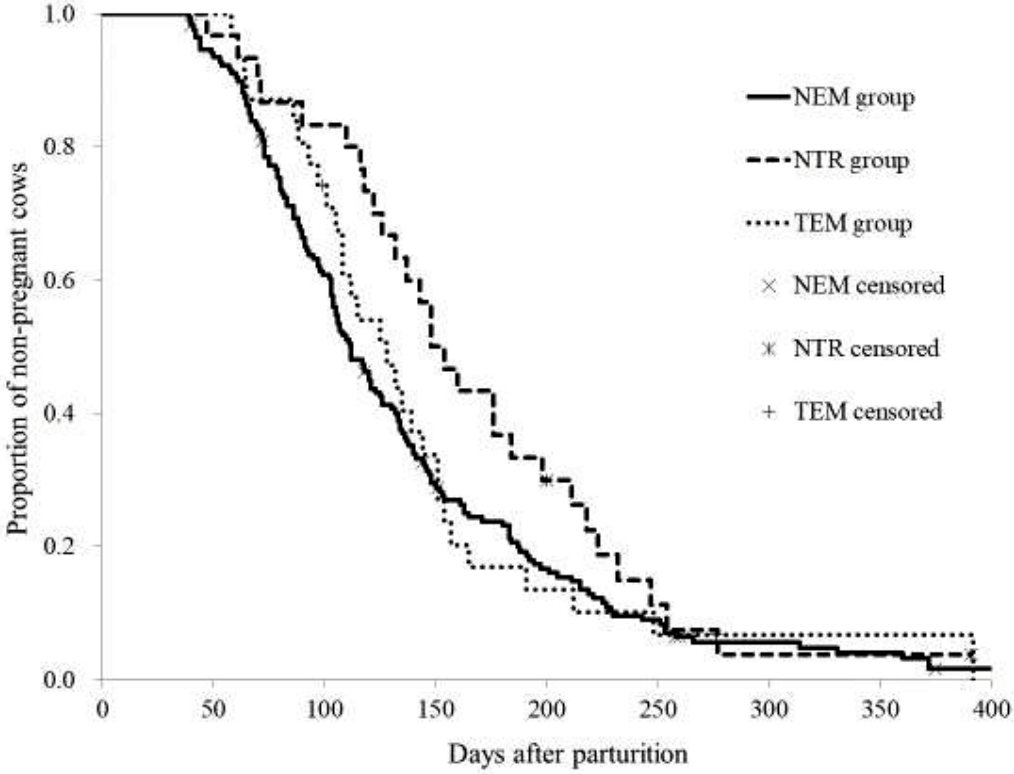
1.0
0.8
0.6
0.4
0.2
0.0
Proportion of non-pregnant cows
0
50
100
150
200
250
300
350
400
Days after parturition
NEM group
NTR group
TEM group
NEM censored
NTR censored
TEM censored

Fig.2

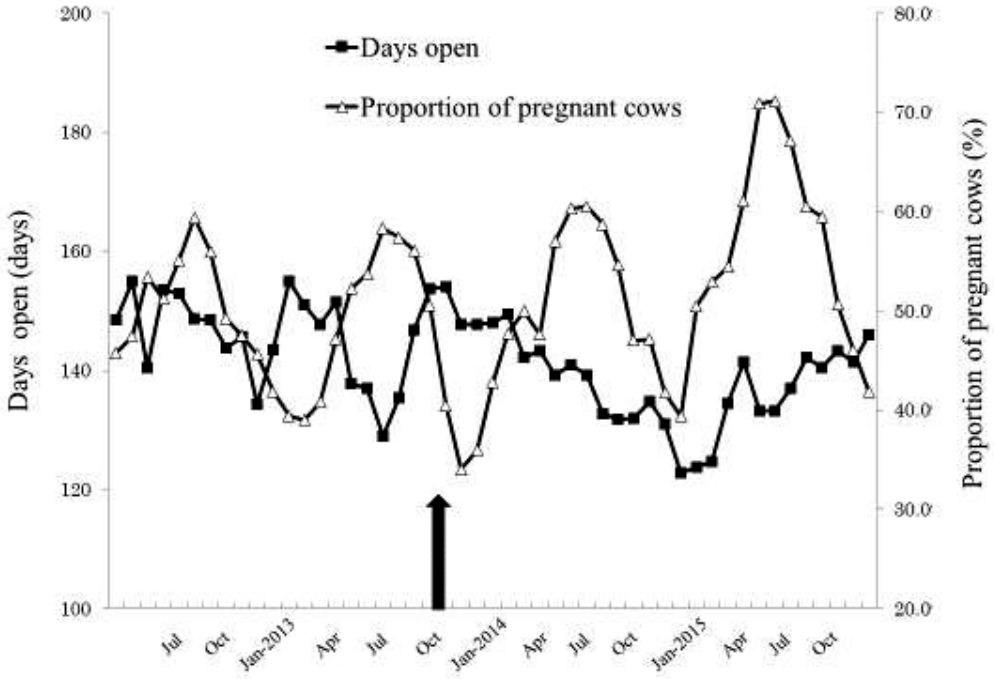
Days open
Proportion of pregnant cows
Days open (days)
Proportion of pregnant cows (%)
200
180
160
140
120
100
80.0
70.0
60.0
50.0
40.0
30.0
20.0
Jul
Oct
Jan-2013
Apr
Jul
Oct
Jan-2014
Apr
Jul
Oct
Jan-2015
Apr
Jul
Oct

Fig.3

Table 1. Prevalence of clinical endometritis at 21 to 60 days after parturition in Holstein cows

Group	VDS	No. of cows classified based on VDS in each interval postpartum at examination (%)			
		21 to 28d (n=31)	29 to 40d (n=102)	41 to 60d (n=96)	Overall (n=229)
NEM	0	18 (58.1)	76 (74.5)	74 (77.1)	168 (73.4)
NTR	1	6 (19.3)	13 (12.8)	11 (11.5)	30 (13.1)
TEM	2	3 (9.7)	9 (8.8)	8 (8.3)	20 (8.7)
	3	4 (12.9)	4 (3.9)	3 (3.1)	11 (4.8)

VDS: Vaginal discharge score

NEM: non-endometritis(VDS=0), NTR: non-treated, mild endometritis(VDS=1), TEM: treated, severe endometritis(VDS=2,3).

Table 2. Reproductive performance outcomes in the three groups treated for the clinical endometritis based on VDS grading system for vaginoscopy in Holstein cows.

Reproductive performance	NEM group	NTR group	TEM group
Parity	2.4±1.3 (n=168)	2.5±1.5 (n=30)	3.0±1.2 (n=31)
Days to FAI	82.6±36.0 (n=165)	89.3±32.9 (n=29)	85.1±26.2 (n=31)
Conception of FAI	78/164 (47.6%)	9/29 (31.0%)	12/31 (38.7%)
No. of AI per pregnancy	2.0±1.3[a] (n=159)	2.8±1.8[b] (n=28)	1.9±0.8[a] (n=29)
Days open	129.5±76.7 (n=159)	155.3±61.3 (n=28)	133.4±66.5 (n=29)

NEM: non-endometritis(VDS=0), NTR: non-treated, mild endometritis(VDS=1), TEM: treated, severe endometritis(VDS=2,3).
a,b : Significant difference ($P<0.05$) between columns.

Table 3. Clinical curing rates (%) and reproductive performance outcomes related to palpable CL or not at treatment and postpartum days at the time of treatment in TEM group in Holstein cows

Reproductive performance	Palpable CL at treatment		Postpartum days at treatment		
	Present	Absent	21-28d	29-40d	41-60d
Clinical cure rate	22/26 (84.6%)	4/5 (80.0%)	9/12 (75%)	13/13 (100%)	9/11 (81.8%)
Calving to FAI interval	87.4 ± 26.5 (n=26)	84.4 ± 26.6 (n=5)	86.1 ± 30.2 (n=7)	74.4 ± 19.7[a] (n=13)	97.2 ± 27.1[b] (n=11)
Conception of FAI	11/26 (42.3%)	1/5 (20.0%)	1/7 (14.3%)	4/13 (30.8%)	6/11 (54.5%)
No. of AI	1.8 ± 0.9 (n=24)	2.0 ± 0.7 (n=5)	2.3 ± 1.0 (n=6)	1.9 ± 0.8 (n=13)	1.5 ± 0.7 (n=10)
Days open	131.3 ± 68.5 (n=24)	146.6 ± 61.7 (n=5)	183.7 ± 111.6 (n=6)	119.5 ± 38.0 (n=13)	123.0 ± 53.3 (n=10)

a,b : Trend towards significant difference ($0.05 < P < 0.1$) between columns

Chapter 2

Risk factors associated with reproductive performance in Japanese dairy cows: Vaginal discharge with flecks of pus or calving abnormality extend time to pregnancy

ABSTRACT

The present study aimed to determine possible risk factors affecting reproductive performance, especially time taken to establish pregnancy in dairy cows, using Cox's proportional hazard model. The data were collected from 154 Holstein Friesian cows (199 lactations). Cows diagnosed with a vaginal discharge score (VDS) of one or calving abnormality showed significantly delayed pregnancy: hazard ratio (HR)=0.654 (95% confidence interval [CI]: 0.436–0.983; P=0.041) and HR=0.457 (95% CI: 0.270–0.774; P=0.004), respectively. Our study suggested that the occurrence of VDS of one or calving abnormality might be possible risk factors increasing the number of days open and affecting reproductive performance in dairy cows.

INTRODUCTION

Optimal reproductive efficiency is a prerequisite for guaranteeing the profitability of the dairy industry. Multiple farm management-related and environmental factors, including perinatal management, affect reproductive performance [Goto *et al.*, 2016]. Previously, we focused on diagnostic methods for clinical postpartum endometritis using a grading system based on vaginal discharge score (VDS), and found that untreated cows having a VDS of one showed a negative reproductive progression [Okawa and Fujikura., 2017]. In addition, we reported that the retention of fetal membranes, as well as other early postpartum conditions within 30 days of parturition, was related to the occurrence of clinical endometritis [Okawa *et al.*, 2017]. However, poor reproductive performance can be caused by conditions other than clinical endometritis, and many other related factors can contribute to postpartum diseases and conditions [Hayes *et al.*, 2012; Hossein-Zadeh, 2010; Sasaki *et al.*, 2014].

Although many studies were conducted on the relationship of clinical endometritis with reproductive performance, whether having a VDS of one is related to negative reproductive prognosis, and whether should be treated or not, have remained controversial until date [Gautam *et al.*, 2009; Leutert *et al.*, 2012; McDougall *et al.*, 2013]. Few studies have focused on the effects of the cows having VDS of one during the early postpartum period, and therefore, the other factors affecting reproductive performance, especially the length of the postpartum interval to established pregnancy need to be investigated [Gautam *et al.*, 2009; Gautam *et al.*, 2010; Giuliodori *et al.*, 2013; Plöntzke *et al.*, 2011; Prunner *et al.*, 2014]. Among many related factors, including the detection of a VDS of one, which affect reproductive performance, it is necessary to identify the

factors that have a greater effect. Therefore, the objective of this study was to determine the possible risk factors affecting reproductive performance, with a focus on the effect of the time needed to establish a successful pregnancy (number of days open) using Cox's proportional hazard model.

MATERIALS AND METHODS

In the present study, the data used were extracted from a data set obtained in our previous study [Okawa *et al.*, 2017]. Records for 154 Holstein Friesian cows (total 199 lactations) that calved during a 26-month-period from September 2013 to November 2015, were used for analysis. The cows (parity, 1–6) were reared in four commercial dairy herds in Fukuoka Prefecture, Kyushu, which is the largest island in southern Japan. The size of the lactating herd varied from 20–50 cows. The herds used was non-seasonal, milked twice a day, and the average annual milk production varied between 8,100–10,900 kg per cow. The voluntary waiting period for each herd was set at 40 days postpartum. The cows were bred by artificial insemination (AI), which was performed by local technicians. Each herd was visited on a monthly basis for a clinical reproductive survey, including treatment of reproductive disorders and pregnancy diagnosis. Pregnancy was diagnosed by transrectal palpation or ultrasonography, 40 days after insemination. For cows that were not inseminated within 90 days postpartum, or cows not pregnant at the time of pregnancy diagnosis, an additional clinical investigation and treatment was performed. In brief, the cows in which estrus was hard to detect (anestrus) were treated by a timed AI program using combined injections of PGF2α and estradiol.

Information for each individual, including the parity number, calving season, status of vaginal discharge based on VDS test, medical records for retained placenta and early postpartum diseases within 30 days of parturition, calving abnormalities including dystocia, stillbirth, and twinning, and the use/absence of a timed AI program within 150 days of parturition, were included in the data used in the present analysis. Parity numbers were classified into four different categories (1, 2, 3, and >4). Calving seasons were

classified into four categories (Spring, March–May; Summer, June–August; Autumn, September–November; Winter, December–February). The VDS test was conducted in all the cows 21–60 days (mean 39.3 ± 9.2) after parturition by using the method for examination described in our previous study [Okawa *et al.*, 2017]. The VDS was used to classify the status of vaginal discharge: 0 =no or clear mucus; 1 =mucus containing flecks of white or off-white purulent material; 2 =discharge containing less than 50% purulent material; and 3 =discharge containing more than 50% purulent material [Leutert *et al.*, 2012; Sheldon *et al.*, 2016]. In the present study, we focused specifically on the effect of VDS of one. Cows with a VDS of two or above were excluded from the analysis. The mean postpartum days of cows with VDS=1 (n=169) and VDS=0 (n=30) at the time of VDS test were 37.2 ± 9.4 and 39.7 ± 9.2 respectively, and the difference was not significant. Medical records on retained placenta and other early postpartum diseases observed in each individual cow within 30 days of the parturition period were obtained from the records of the Federation of Agricultural Mutual Relief Association. Individuals that retained placenta, defined as the retention of fetal membranes >24 hr after parturition, were administrated an intramuscular PGF2α injection within a period of seven days after parturition. Early postpartum diseases observed within 30 days parturition included milk fever, puerperal fever (metritis), hyperketonemia (ketosis), and acute mastitis. Dystocia was defined as a calving that required veterinary assistance. Stillbirth was defined as a dead fetus found at calving or immediately after calving. Information on the calving conditions and hence information on productivity was obtained from the Livestock Improvement Association of Japan (LIAJ). A limited number of cows (n=54) used in this study underwent a timed AI program within 150 days of parturition because of anestrus. The intramuscular administration of 2 mg of estradiol benzoate (Ovahormon®; ASUKA

Animal Health Co., Ltd., Tokyo, Japan) and insertion of a controlled internal drug-releasing device (CIDR; CIDR® 1900; Zoetis Japan Co., Ltd., Tokyo, Japan), were conducted on day 0; the intramuscular administration of 500 μg of cloprostenol (Estrumate®; Intervet K.K, Tokyo, Japan) was performed on day 9, along with CIDR removal; 1 mg of estradiol benzoate was administered on day 10 and was followed by AI on day 11.

All statistical analyses were performed using the Bell Curve for Excel software program (Social Survey Research Information Co., Ltd., Tokyo, Japan). The dates regarding the time to establish the pregnancy were analyzed using Cox's proportional hazards regression model. Pregnancy was set as an endpoint, and the culling rate owing to infertility or post-partum diseases was set as censoring. The explanatory variables used were parity number, calving season, VDS of one, retained placenta and early postpartum diseases, calving abnormality, and occurrence of a timed AI program within 150 days of parturition. Each explanatory variable was initially examined using univariate analysis. The associated variables ($P<0.1$) were used for multivariable analysis based on the Cox's proportional hazards regression model of time to pregnancy. In addition, significant variables of multivariable analysis were visualized using Kaplan-Meier survival curves, and the differences were analyzed using a generalized Wilcoxon test. $P<0.05$ was considered statistically significant.

RESULTS

Univariate analysis selected parity, season of calving, VDS of one, retained placenta, and calving abnormality for multivariable analysis (Table 1). The multivariable analysis showed that VDS of one (hazard ratio=0.654; P=0.041) and calving abnormality (hazard ratio=0.457; P=0.004) were significantly associated with the number of days open postpartum to establish the pregnancy (Table 2). Moreover, in the survival analysis (Fig. 1), both VDS of one and calving abnormality increased the time to pregnancy significantly (P=0.018 and 0.0096, respectively) as compared with that of the control group.

DISCUSSION

Consistent with the findings of our previous study [Okawa *et al.*, 2017], the cows with a VDS of one included in the present study showed a reduced reproductive performance. Although the occurrence of clinical endometritis was shown to be associated positively with the retention of fetal membranes and early postpartum diseases within 30 days of parturition in our previous study [Okawa and Fujikura, 2017], the findings of this study indicate that calving abnormality and VDS of one were associated with extending the number of days open postpartum to establish a pregnancy.

In Japan, especially in the experimental area, the average calving to conception interval in dairy cows is approximately 160 days [LIAJ, 2017] and the median calving to conception interval in dairy cows is approximately 177 days [Goto *et al.*, 2016]. Although the use of a timed AI program within 150 days after parturition was generally considered an economically important factor that could improve reproductive performance, no association of the timed AI program with the number of days open was observed in the present study. Conditions such as stillbirth and dystocia have negative effects on reproductive performance [Gaafar *et al.*, 2011; Sasaki *et al.*, 2014], and therefore, milk production [Gaafar *et al.*, 2011]. In addition, Hossein-Zadeh reported that dairy cows that gave birth to twins had an increased number of days open as compared with those that gave birth to a single calf (129.28 days vs. 144.88 days) [Hossein-Zadeh, 2010]. In dairy management, the effective prevention of twinning might be difficult, because parity number and level of milk production appear to be positively associated with increased ovulation rate, and therefore, twinning rate [Fricke and Wiltbank, 1999; Kinsel *et al.*, 1998; Wiltbank *et al.*, 2000]. In addition, twinning is positively associated with a higher

risk of dystocia and increased neonatal mortality [Gaafar *et al.*, 2011; Silva del Rio *et al.*, 2007]. In the present study, we have shown the negative effect of calving abnormality and VDS of one on the postpartum time to establish a pregnancy. Although calving abnormality had no significant association with the risk of clinical endometritis, as shown in one of our previous studies [Okawa and Fujikura, 2017], the present study indicated that calving abnormality negatively affected reproductive performance. These findings suggest that cows that experienced calving abnormality have potential risk factors, such as subclinical endometritis, due to the abnormal intrauterine environment [Hoseein-Zadeh, 2010; Salasel *et al.*, 2010; Sasaki *et al.*, 2014].

Unfortunately, in this study, we did not examine and diagnose definitive endometritis by scoring endometrial cytology, which is the most definitive and reliable method of doing so [de Boer *et al.*, 2014; Leutert *et al.*, 2012; Sheldon *et al.*, 2006]. Therefore, in cows that experienced calving abnormalities, the intrauterine environments should be examined using endometrial cytology, and treated accordingly. To address this issue, dairy herdsmen and veterinary practitioners should pay special attention to assistance during calving, particularly in cases of dystocia or twinning. Considering potential risks of reduced fertility, it is vital to perform appropriate diagnosis and treatment for recovering uterine conditions. In the early postpartum period, it is well known that the uterine conditions change rapidly with spontaneous resolution. Therefore, vaginal discharges might constitute a part of the physiological self-cleaning process [Gautam *et al.*, 2010; Sheldon *et al.*, 2006]. Although vaginal discharge is an important part of symptoms of clinical endometritis, it does not necessarily reflect endometrium inflammation, and it indicates that some cows have the possibility of cervicitis without endometritis [Hartmann *et al.*, 2016; Westermann *et al.*, 2010]. In the present study, VDS

test was performed at 21–60 days after parturition. Moreover, we diagnosed cows with a VDS of one or above for clinical endometritis under field conditions [Okawa and Fujikura, 2017; Okawa *et al.*, 2017]. As mentioned above, uterine conditions during early postpartum period change rapidly. Therefore, for a more definitive and reliable diagnosis of clinical endometritis, evaluation of VDS test during the postpartum must be interpreted based on days (weeks) postpartum, and may be used in combination with endometrial cytology.

In conclusion, the occurrences of VDS of one or calving abnormality postpartum in dairy cows were shown to be possible risk factors for extending the number of days open, and consequently, a reduced reproductive performance of the herd. Previous studies have reported that other factors (e.g., housing system, mastitis, somatic cell count, and claw health) also affect reproductive performance in dairy herds negatively [Fuenzalida *et al*, 2015., Gautam *et al.*, 2009; Lomander *et al.*, 2013]. Future studies using a larger number of cows and farms, aimed at identifying the relationship of management with environmental factors and reproductive performance, are necessary under conditions relevant to Japan.

FIGURE LEGENDS

Fig. 1. (A) Kaplan-Meier survival curves for the population of open cows with no vaginal discharge (vaginal discharge score [VDS] = 0) or VDS = 1. Cows with no vaginal discharge: solid line, n = 169; median open days = 112; 5.3% (n = 9) of the cows studied were censored. Cows with VDS of 1: broken line, n = 30; median open days = 148; 6.7% (n = 2) of the cows studied were censored. (B) Kaplan-Meier survival curves for the population of open cows with normal calving and calving abnormality. Cows with normal calving: solid line, n = 181; median open days = 118; 5.5% (n = 10) of the cows studied were censored. Cows with calving abnormality: broken line, n = 18; median open days = 183; 5.6% (n = 1) of the cows studied were censored.

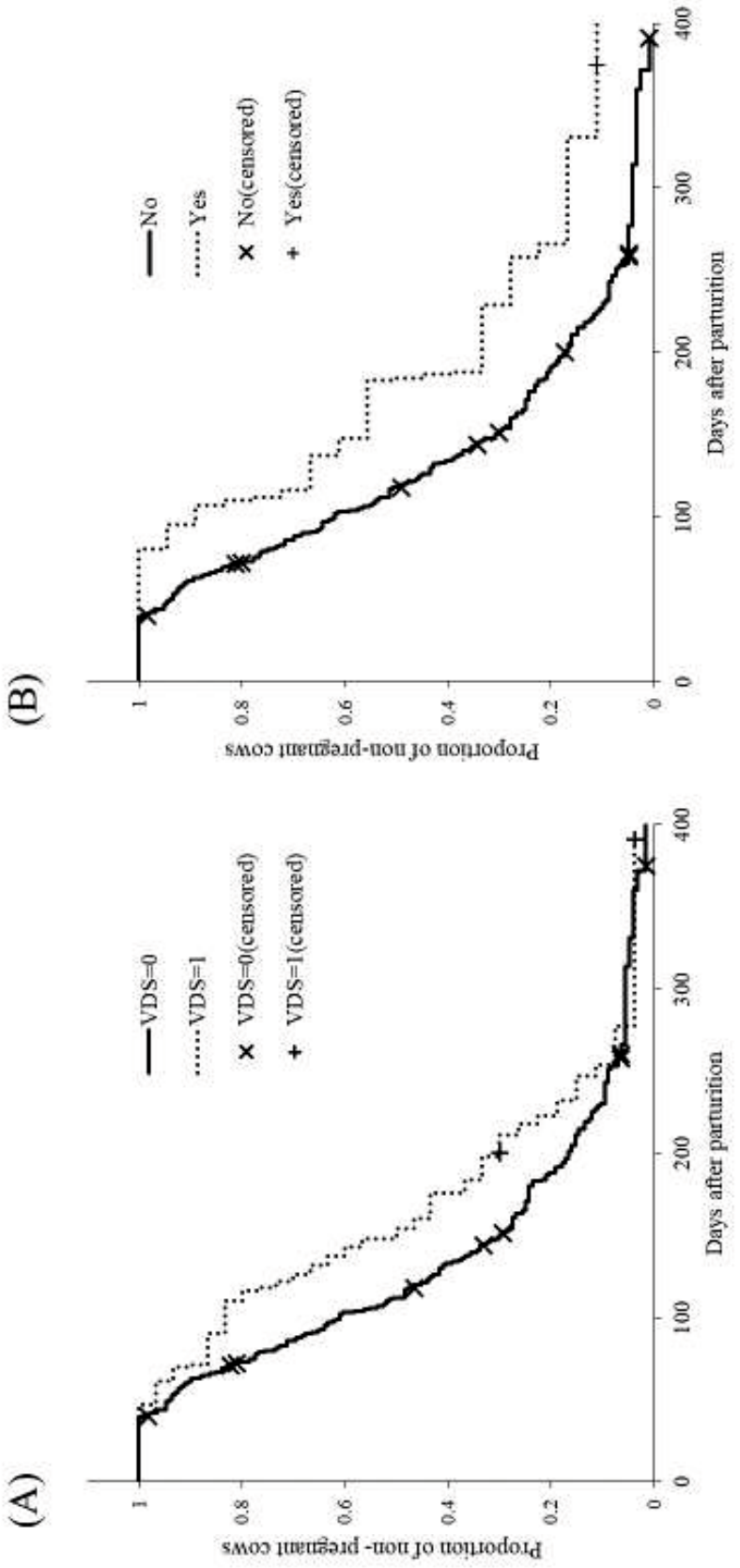
(A)
Proportion of non- pregnant cows
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
Days after parturition
VDS=0
VDS=1
VDS=0(censored)
VDS=1(censored)
(B)
Proportion of non-pregnant cows
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
Days after parturition
No
Yes
No(censored)
Yes(censored)

Figure.1

Table.1 Result of univariate analysis with Cox's proprortional hazards model of time to pregnancy.

Variable	Category	n	HR time to pregnancy*	95% CI	*p*-value
Parity	1	60	Reference	-	-
	2	56	0.682	0.467-0.995	0.047
	3	41	0.984	0.648-1.494	0.940
	> 4	42	0.738	0.490-1.110	0.144
Season	Spring	30	Reference	-	-
	Summer	50	1.104	0.681-1.790	0.690
	Autum	76	1.248	0.805-1.935	0.322
	Winter	43	0.593	0.357-0.987	0.044
Mild vaginal discharge	No (VDS=0)	169	Reference	-	-
	Yes (VDS=1)	30	0.68	0.454-1.017	0.06
Retention of fetal membranes	No	191	Reference	-	-
	Yes	8	0.497	0.220-1.125	0.093
Postpartum disease within 30 DIM	No	161	Reference	-	-
	Yes	38	1.315	0.906-1.908	0.15
Calving abnormality	No	181	Reference	-	-
	Yes	18	0.498	0.296-0.836	0.008
Timed AI program within 150 DIM	No	145	Reference	-	-
	Yes	54	1.256	0.911-1.732	0.164

*HR time to pregnancy: Hazard ratio time to pregancy

Table.2 Result of multivariate analysis with Cox's proportional hazards model of time to pregnancy.

Variable	Category	Cox's proportional hazards model		
		HR time to pregnancy*	95% CI	*p*-value
Calving abnormality	No	reference		0.004
	Yes	0.457	0.270-0.774	
Retention of fetal membranes	No	reference		0.059
	Yes	0.452	0.199-1.029	
Mild vaginal discharge	No (VDS=0)	reference		0.041
	Yes (VDS=1)	0.654	0.436-0.983	

*HR time to pregnancy: Hazard ratio time to pregancy

OVERALL DISCUSSION

For veterinary practitioners specializing in farm animals, effective and definitive diagnosis is important. However, there are many limitations and considerations, including costs, sampling time, on-farm convenience of the method, requirement for laboratory skills, and time to report. With these limitations in mind, it is important to choose effective diagnostic and treatment methods in clinical practice.

Bovine endometritis is an important disease in dairy cows, and clinical endometritis is defined as the presence of a purulent vaginal discharge detectable in the vagina 21 days or more postpartum, while subclinical endometritis is characterized by inflammation of the endometrium in the absence of signs of clinical endometritis. Subclinical endometritis cannot be diagnosed using vaginoscopy and requires cytology [Sheldon *et al*., 2009]. It follows that cows with mild or no clinical endometritis may have subclinical pathology in their uterus. In addition, it is not only clinical endometritis that leads to decreased fertility in dairy cows in Japan; many related factors contribute to reproductive performance.

In Chapter 1 of the present study, we demonstrated that cows with clinical endometritis (VDS ≥1) treated using PGF2α recovered and regained fertility more quickly than cows with no treatment having mild clinical endometritis (VDS of 1). In addition, early treatment (29–40 days postpartum) may have a positive effect on the management of dairy herds. Conversely, cows that received no receive treatment for their mild endometritis (VDS of 1) showed low reproductive efficiency. One previous study

suggested that mild endometritis with a VDS of 1 may have no adverse effects on reproductive performance, and that a small amount of vaginal discharge in the early postpartum period may be part of the physiological self-cleaning process [Gautam *et al*., 2010; Hirsbrunner *et al*., 2006]. Our data in Chapter 1 suggested that cows with mild endometritis require treatment to recover uterine condition and fertility, similar to cows with severe endometritis. Vaginoscopy is not a perfect method for diagnosing endometritis because it has only moderate sensitivity. However, it may be suitable as a field diagnostic method in clinical practice [de Boer *et al*., 2014; Gautam *et al*., 2009; Gautam *et al*., 2010.; Leutert *et al*., 2012].

According to the clinical guidelines of the Agricultural Disaster Compensation System of the Ministry of Agriculture, Forestry, and Fisheries of Japan, the initiation date of treatment for cows diagnosed with reproductive disorders is limited to 40 days postpartum or later [Ministry of Agriculture, Forestry, and Fisheries, 2014]. It is not clear what scientific evidence was used to support this limitation, and the guidelines have not been revised since 1999. We demonstrated that PGF2α treatment carried out 29 to 40 days postpartum tended to confer a shorter calving to FAI interval than treatment carried out 41 to 60 days postpartum. This indicates that treatment of clinical endometritis before 40 days postpartum may improve reproductive performance in Japanese dairy cows.

We could not clarify whether CL status influenced the clinical cure rate and reproductive performance after PGF2α treatment. In cows, the ovarian and uterine conditions vary drastically with postpartum days. Therefore, to avoid confounding, further studies using stratified analyses are needed to assess the effect of PGF2α

administration on uterine resumption.

We previously reported that fetal membrane retention and other early postpartum diseases within 30 days postpartum were related to clinical endometritis [Okawa and Fujikura, 2017]. In Chapter 2, the findings indicated that calving abnormalities and VDS of 1 were associated with an increased number of days open postpartum to establish pregnancy. Interestingly, calving abnormalities had no significant association with the risk of clinical endometritis in our previous study, but negatively affected reproductive performance in Chapter 2 of the present study. These findings suggest that cows experiencing calving abnormalities may have risk factors, such as subclinical endometritis, because of an abnormal uterine environment [Hossein-Zadeh, 2010; Salasel *et al*., 2010; Sasaki *et al.*, 2014]. Therefore, to improve reproductive performance in clinical practice in Japan, it may be necessary to select cows that have experienced an abnormal event during the puerperal period, such as abnormal calving and fetal membrane retention, for examination using endometrial cytology.

In conclusion, the above findings indicated that a grading system based on VDS can be used to diagnose clinical endometritis in routine, cow-side reproductive practice, and that early treatment of clinical endometritis using PGF2α administration within 40 days postpartum may improve reproductive performance. In addition, using Cox's proportional hazard model, we determined that a VDS of 1 or calving abnormalities postpartum are possible risk factors for an increased number of days open in dairy cows.

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