Hormonal Evaluation for Reproductive Management and Treatment with Anti-viral Drug and Epidemiological Study for Equine Coital Exanthema in Thoroughbred Stud

サラブレッド種馬所における内分泌学的評価を用いた繁殖管理及び馬媾疹に対する抗ウイルス薬による治療と疫学調査

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CONTENTS

ABBREVIATIONS

ALP	alkaline phosphatase
CLEIA	chemiluminescent enzyme immunoassay
CPE	cytopathic effect
E	estradiol
EC_{50}	50% effective concentration
ECE	equine coital exanthema
EHV-3	equine herpesvirus 3
EIA	enzyme immunoassay
FCS	fetal calf serum
FHK	fetal horse kidney

FIA	fluoroimmunoassay
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotropin
IHC	immunohistochemical
i.m.	intramuscular
ISBC	the International Stud Book Committee
JAIRS	Japan Association for International Racing and Stud Book
LH	luteinizing hormone
MEM	minimum essential medium
P	progesterone
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PGF2α	prostaglandin F2α
RIA	radioimmunoassay
SEM	standard error of the mean
SN	serum neutralization
STD	sexually transmitted disease

PREFACE

Thoroughbred has been bred for racing from early 18 centuries in the world. In Japan, Thoroughbred breeding started in 1907 and it is the most popular breed in horses in the present day. In the report of 2014, there are 40,794 Thoroughbreds among 73,977 total horse population in Japan that includes 5,880 heavy breeds, 672 ponies, 1,817 native horses, 9,081 fattening horses and 15,475 horses for riding [1]. More than 6,000 Thoroughbreds are born every year [1]. The pedigree of Thoroughbreds is well managed in each country. In Japan, it is managed by Japan Association for International Racing and Stud Book (JAIRS), Japanese representative of the International Stud Book Committee (ISBC), and all Thoroughbred mares and stallions for breeding are registered with JAIRS. In 2017, 10,320 mares and 267 stallions are registered as broodmare and sire, of those, 97.5% of mares and 93.6% of stallions are managed in Iburi and Hidaka areas in Hokkaido, Japan.

Management of stallions in good health and reproductive conditions and

carrying out mating safely are required for stud. Diagnosis of the estrus condition of mares and diagnosis of reproductive abnormality in stallions are usually done based on clinical findings, but often need special examinations that include evaluation of endocrine status of mares and stallions. However, hormone assays are expensive and time-consuming. Further, there were no commercially available sex steroid hormone assay systems that is verified for horse use. Therefore, in Chapter I, application of a hormone assay system developed for human use, that is inexpensive and gives results in short time, was verified for evaluation of the reproductive conditions in mares and stallions.

In Thoroughbred horse racing industry, all horses are bred by natural service since the rule set by ISBC [108]. However, mares and stallions for breeding are selected by their race results and pedigree, not by their fertility. Therefore, some stallions are found to be infertile after registration to JAIRS as sire. Sometimes there are stallions with cryptorchidism, without testes in scrotum, are diagnosed by evaluation of testosterone (T) concentrations after human chorionic gonadotrophin (hCG) administration. On the other hands, some stallion shows low libido since young males are managed separately from females and trained not to show an interest to females during the training period for racing [57]. This may make some stallions hesitate to mount mares in estrus when they are introduced to breeding studs. Thus, differential diagnosis is needed to segregate causes of mating difficulty due to these mental reasons from physical reasons and functional abnormalities of genital organs. Hormonal evaluation is again useful for this usage.

For female aspects, there are also some reproductive problems. Horses are

long-day breeder and breeding season is spring and summer [82]. However, producers often prefer to start breeding early months (February ~ March) of the year since foals that were born early months of the year are large in body size and sell better than the smaller foals that are born in late months of the year. For early breeding, the length of day-time is controlled by artificial light and some hormonal products are used to activate the estrus cycle in mares [14]. Hormone assays are useful tools to monitor ovarian activities especially during early months of the year. Further, most farms managing mares do not have a male horse as a teaser and timing of mating is usually diagnosed by rectal palpation and ultrasonography. Consequently, some mares with poor estrus conditions, too early or too late for breeding, are sent to breeding studs. Again, hormone assays are helpful to evaluate the ovarian activities of mares.

Some automated benchtop analyzers for measuring steroid hormone concentrations are available for human. One of those system, a chemiluminescent enzyme immunoassay (CLEIA) system (PATHFAST, LSI Medicine Corporation, Tokyo, Japan) was developed for measurement of hormonal concentrations in human [18, 52, 91]. This system can measure sex steroid hormones in serum and whole blood without extraction within 30 minutes. However, binding proteins and other factors in sera often interfere in immunoassay systems; thus, an immunoassay system developed for one species needs to be verified for other species and sometimes for other applications [20]. There is no report for verification to use these systems for horses.

In chapter II, two clinical cases of equine coital exanthema (ECE), a

sexually transmitted disease (STD) caused by equine herpesvirus 3 (EHV-3), are described together with a sero-epidemiological study of ECE. Prevention and control of STD is another important aspect of stud management. The laws (i.e. Act on Domestic Animal Infectious Diseases Control and Act on Improvement and Increased Production of Livestock) defined some diseases including equine infectious anemia, equine paratyphoid, contagious equine metritis and equine rhinopneumonitis, that must be controlled by culling or reporting to the local authorities. Recently, ECE caused by EHV-3, a disease not in the lists of diseases of the laws diagnosed in our stud sporadically. There had been no epidemiological study on ECE in Japan. Stallions affected by ECE cannot mate with mares for pain and the risk of transmission [2, 6, 9, 29]. Thus, ECE causes a serious economic loss of breeding studs and farms. Acyclovir, an anti-herpesvirus agent, has been shown an effective to ECE [6, 19, 81]. Valacyclovir, a prodrug of acyclovir, has been used for treatment of equine rhinopneumonitis caused by equine herpesvirus 1 and shown its effectiveness [32] partly because due to, a longer half-life in circulation [33].

This study is composed of two chapters. In Chapter I, the application of the CLEIA system for sex steroid hormone assays in horses was verified. In Chapter II, the clinical cases of ECE were investigated and a sero-epidemiological survey for ECE was conducted in Japan.

CHAPTER I Verification and application of chemiluminescent enzyme immunoassay system for diagnosis of reproductive status in horses

Section 1. Progesterone

1.1.1. Introduction

The physiological function of progesterone (P) is to prepare the uterine endometrium for implantation of embryos [37, 44] and maintain uterine conditions for the growing fetus during pregnancy in females [40]. Therefore, measurement of P is useful for detecting luteal function [3] and placental function in cyclic and pregnant mares. There is a need to rapidly measure P in equine clinical sites for early diagnosis of luteal activity during the estrous cycle. It is also important to know the levels of circulating P of estrous mares before covering with stallions to get normal fertilization and growing embryos.

Chemiluminescent enzyme immunoassay (CLEIA) system (PATHFAST, LSI Medience Corporation, Tokyo, Japan) was developed for human use as a small, automated, bench top analyzer [18, 52, 91]. This system can measure sex steroid hormones in sera and whole blood within 30 minutes (min) without extraction using a dedicated cartridge. All procedures are done automatically and it does not need to set up standard curve on each measurement. The cost of using the CLEIA system is equal to or lower than that of the existing radioimmunoassay (RIA), the enzyme immunoassay (EIA) and the fluoroimmunoassay (FIA). Unlike proteinaceous gonadotropins, structures of steroid hormones are not different between species. However, binding proteins and other factors in sera often interfere in immunoassay systems; thus, an immunoassay system developed for one species needs to be verified for other species and, sometimes, for other applications [20].

The purpose of this study was to evaluate the validity of CLEIA system by comparing the P concentrations measured by this system with those measured by RIA and EIA which have been used in mares. Then, P measurement by the CLEIA system using whole blood as a sample was verified for field use.

1.1.2. Materials and Methods

Animals

Mares in Iburi area, Hokkaido, Japan, kept under natural conditions were used for measuring circulating P. Three Thoroughbred mares (4-11 years old) were used for comparison of P concentrations measured by CLEIA system and RIA, and in whole blood and serum. Four Thoroughbred mares (4-11 years old) were used for comparison of P concentrations measured by CLEIA system and EIA. All mares were subjected to daily ultrasonographic investigation using a B-mode scanner (ALOKA SSD-620, Hitachi Medical Corporation, Tokyo, Japan), and their ovaries were monitored. After confirming the existence of a corpus luteum, all mares were administrated a single intramuscular (i.m.) injection of 1 ml (250 μg) of a synthetic analogue of prostaglandin F2α (PGF2α, Estrumate, Intervet, Tokyo, Japan). Blood samples were then collected from the jugular vein at 0, 15, 30 and 45 min, at one-hour (hr) intervals until 24 hr and at 48 hr via a catheter in the jugular vein for comparison of P concentrations measured by CLEIA system and RIA, and in whole blood and serum (n = 88). Blood samples were collected with the same way until 24 hr for comparison of P concentrations measured by CLEIA system and EIA (n = 112).

Sample preparation and experiments

For collection of serum samples, whole blood was drawn from the jugular vein of the mares into a plain blood collection tube. Serum was separated by centrifugation at 1,700 ×*g* for 10 min. For the correlation experiments between whole blood and serum, whole blood samples were collected from the jugular vein into heparin sodium-loaded tubes. Concentrations of P in all samples were measured by CLEIA system. Serum samples were used for comparisons between CLEIA system and RIA or EIA. All procedures were carried out in accordance with the guidelines established by the institutional Animal Care and Use Committee of Tokyo University of Agriculture and Technology.

Hormone assay

1) CLEIA system

P concentrations in serum and whole blood were measured by a CLEIA system that was developed and verified for human sera, plasma, and heparinized whole blood. 100 μl of either serum or whole blood was applied to a reagent cartridge for the P assay (PF0181-K, LSI Medience Corporation) without extraction. The reagent cartridge was set into a CLEIA analyzer to run assays. All reagents were contained in the cartridge, and all of the procedures of the assay were performed inside it. The CLEIA system is based on a one-step competitive immunoassay system method. Using this procedure, samples were first mixed with an alkaline phosphatase (ALP)-labeled antibody and a magnetic latex reagent.

Magtration® was then used to remove excess reagents and residual materials not bound to the magnetic latex. A chemiluminescent substrate (CDP-Star®) was added, and luminescence was emitted upon binding to the ALP. Luminescence was then measured and hormonal concentrations were determined. The assay range of CLEIA system was 0.2-40.0 ng/ml. Based on the manufacturer's catalogue, the intra-assay coefficients of variance were determined to be 3.61%-10.23% for serum and 7.1% -16.7% for whole blood samples.

2) RIA and EIA

Serum concentrations of P were determined by a double-antibody RIA system using ^{125}I-labeled radioligands as described previously [99]. 500 µl of standard and sample were extracted for P once with 2 ml of ether. After extraction, 300 µl of phosphatase buffered saline (PBS, 0.05M) containing 1% bovine serum albumin (BSA) was added. 100 µl of standard or sample was mixed with 100 µl of anti-progesterone (GDN337, provided by Dr. G. D. Niswender, Animal Reproduction and Biotechnology, Colorado State University, Fort Collins, Co, U.S.A.) in PBS (0.05M) containing normal sheep serum and EDTA (0.05M). And then, 100 µl of ^{125}I-labelled radioligands in PBS (0.05M) containing 1% BSA was added and incubated at 4°C for 24 hr. After incubation, 100 µl of anti-sheep gamma globulin diluted with PBS (0.05M) containing 5% polyethylene glycol was added and incubated at 4°C for 24hr. After that, standard and samples were centrifuged at 1700 ×g for 30 min at 4°C. Supernatant was removed and radioactivity was

counted by gamma-counter. The assay results were obtained within 48 hr. The intra- and inter-assay coefficients of variance were 6.3% and 7.2%.

Serum concentrations of P were also determined by a double-antibody EIA system without ether extraction as described previously [70]. To prepare immuno plate, 100 μl of anti-rabbit-IgG (H+L)-goat (Jackson ImmunoResearch; 111-005-003) diluted to 2 μg/ml was added to 96 well immuno plate (Nunc; 442404) and the plate was incubated at room temperature for 24 hr. Anti-rabbit-IgG not combined to plate was removed and 250 μg of assay buffer including 0.1% of BSA was added. The plate was incubated at room temperature for 30 min and this plate was stored at 4°C until assay. For measurement of P concentration, 20 μg of standard for P and samples were added to each well. 100 μl of anti-progesterone rabbit serum diluted 100,000 times and 100 μl of P labeled with Horseradish Peroxydase were added and the plate was incubated at 4°C overnight. After incubation, supernatant was removed and the plate was washed with 0.05% of Tween 80 for 4 times. 150 μg of substrate buffer was added and incubated at 35°C for 40 min. After incubation, 50μg of $4NH_2SO_4$ was added and absorbance at 450 nm was measured (BIORAD Model 450 Microplate Reader). The assay results were obtained within 48 hr. The intra- and inter-assay coefficients of variance were 6.3% and 10.6%.

Statistics

Pearson's r was calculated to find correlation between variables using the GraphPad Prism 5 software for Windows (GraphPad Software, San Diego, CA, U.S.A.).

1.1.3. Results

The concentrations of P in serum samples measured by CLEIA system were compared with those measured by RIA (Fig. 1.1.1). The results obtained by CLEIA system showed a significant correlation with those obtained by RIA ($r = 0.9654$, $p < 0.0001$, $n = 88$). The pattern of circulating P in serum obtained by CLEIA system showed excellent correlation with that obtained by RIA, although the values measured by CLEIA system were about two times higher than those measured by RIA (Fig. 1.1.2).

In addition, the concentrations of P in serum samples measured by CLEIA system were also compared with those measured by EIA (Figs. 1.1.3 and 1.1.4). A high positive correlation was observed between the concentrations of P measured by CLEIA system and EIA (Fig. 1.1.3, $r = 0.9323$, $p < 0.0001$, $n = 112$). The concentrations of P in the serum of the four mares measured by CLEIA system showed a parallel pattern to those measured by EIA (Fig. 1.1.4).

The correlation of concentrations of P in whole blood and serum samples measured by CLEIA system was examined (Fig. 1.1.5). There was excellent positive correlation between whole blood and serum samples ($r = 0.9672$, $p < 0.0001$, $n = 88$).

The patterns of circulating P in whole blood and serum measured by CLEIA system were compared in three mares after the single injection of PGF2α (Fig. 1.1.6). Significant correlation of concentrations of P in whole blood and serum samples was observed in all mares. Circulating P in whole blood and serum

samples declined rapidly and had declined by about 50% at 2 hr after the PGF2α injection; the levels reached the basal levels in whole blood (a, 1.07 ng/ml; b, 1.69 ng/ml; c, 1.66 ng/ml) and serum (a, 0.84 ng/ml; b, 1.33 ng/ml; c, 1.36 ng/ml) at 24 hr after injection. A further decline in concentrations of P was observed in both whole blood and serum samples at 48 hr after injection.

1.1.4. Discussion

The present study evaluated the validity of rapid measurement of equine P using a new practical assay system, CLEIA system.

In the experiment in which CLEIA system was compared with RIA and EIA, CLEIA system showed excellent correlation, with correlation coefficients of 0.9654 (n = 88) and 0.9323 (n = 112). In the present study, the concentrations of P in serum of mares at luteolysis measured by CLEIA system were about two times higher than those measured by RIA. Although the exact reason for this is not clear at the present time, it seems to be related to the different methods of sample preparation, such as extraction of steroid hormones using ether in RIA. In addition, the different cross-reactivities of the anti-P sera used in CLEIA system and RIA may affect estimation of the concentrations of P. It does not seem that the difference of the values between two methods reduces the utility of CLEIA system for diagnosis whether the mare coming to stud is in estrus or in luteal phase.

Measurements of P in whole blood and serum samples of mares showed excellent correlation in the present study, indicating that concentrations of P can

be measured in whole blood as well as serum samples in mares using CLEIA system.

The parallelism of the pattern of P levels in serum samples measured by CLEIA system and RIA was confirmed. This is also true for CLEIA system and EIA. These results clearly demonstrated that CLEIA system correctly monitored the physiological changes in circulating P during induced luteolysis in cyclic mares. Physiological changes in circulating P during the process of induced luteolysis in mares were examined using CLEIA system. Measurement of P in whole blood and serum samples showed excellent parallel patterns, indicating that CLEIA system can be used to measure circulating P in whole blood in mares.

In clinical application of P measurement to diagnosis of reproductive condition in the equine clinic, there is a need for a rapid measurement system in addition to a measurement system that uses whole blood samples. In the present study, the concentrations of P in both whole blood and serum samples could be measured by CLEIA system within 30 min without ether extraction. In conclusion, CLEIA system is useful in the equine hospital as an accurate diagnostic tool for rapid assay of P [105].

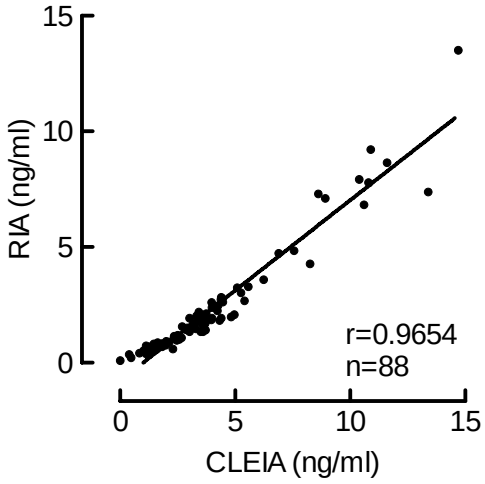

Figure 1.1.1 Correlation plots between progesterone (P) measured by a chemiluminescent enzyme immunoassay (CLEIA) and a radioimmunoassay (RIA) in serum samples.

P concentrations measured by two methods were strongly correlated (r = 0.9654).

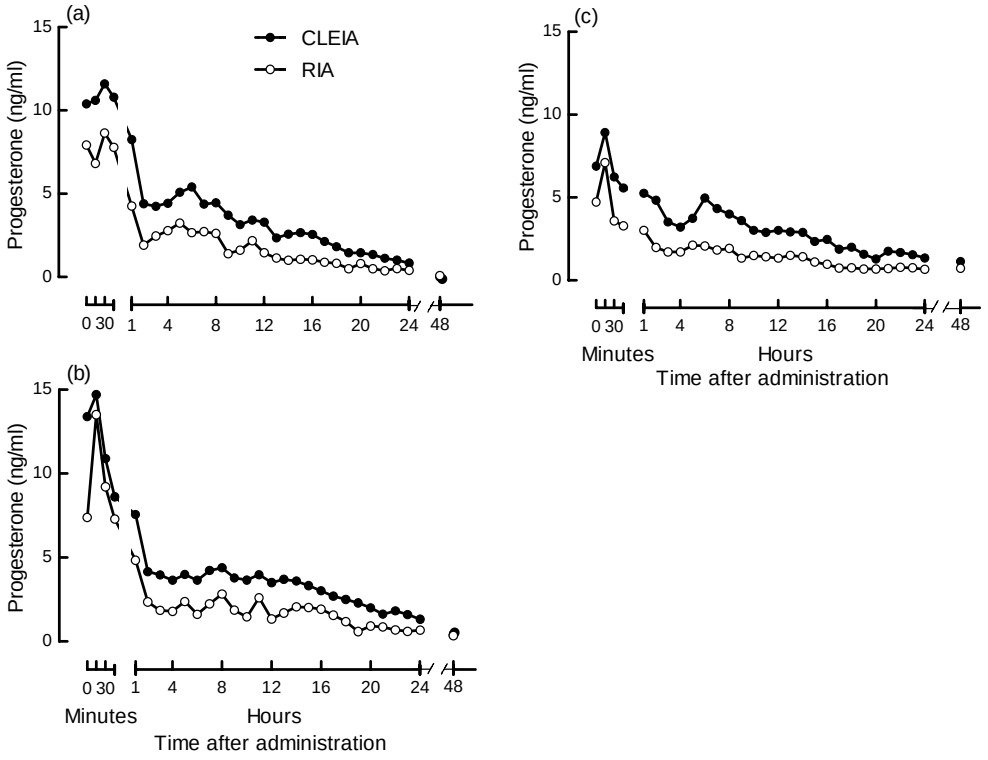

Figure 1.1.2 Changes in circulating progesterone (P) in serum samples of three mares (a, b, c) after administration of a single i.m. injection of prostaglandin F2α.

P concentrations were assayed by a chemiluminescent enzyme immunoassay (CLEIA) (●) and a radioimmunoassay (RIA) (○).

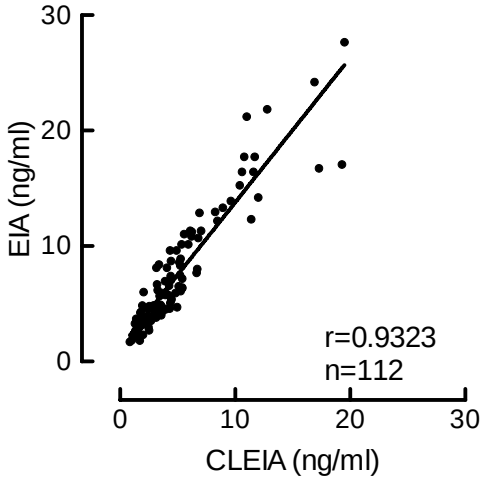

Figure 1.1.3 Correlation plots between progesterone (P) measured by a chemiluminescent enzyme immunoassay (CLEIA) and an enzyme immunoassay (EIA) in serum samples.

P concentrations measured by two methods were strongly correlated (r = 0.9323).

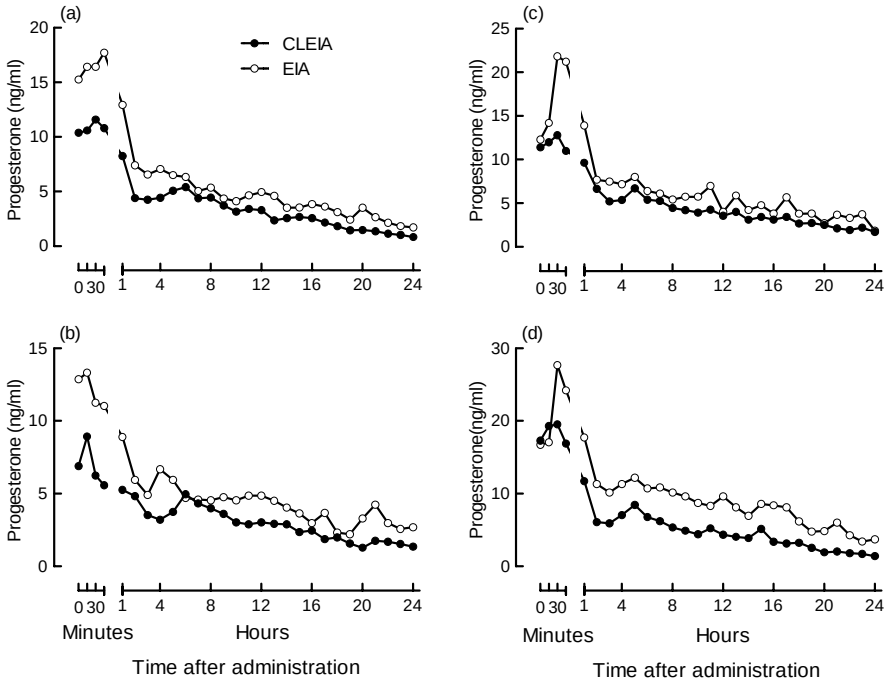

Figure 1.1.4 Changes in circulating progesterone (P) in serum samples of four mares (a, b, c, d) after administration of a single i.m. injection of prostaglandin F2α.

P concentrations were assayed by a chemiluminescent enzyme immunoassay (CLEIA) (●) and an enzyme immunoassay (EIA) (○).

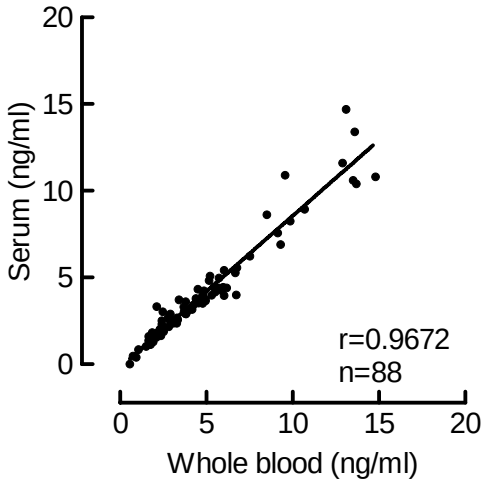

Figure 1.1.5 Correlation plots between progesterone (P) measured by a chemiluminescent enzyme immunoassay in whole blood and serum samples of mares.

P concentrations in whole blood and serum were strongly correlated (r = 0.9672).

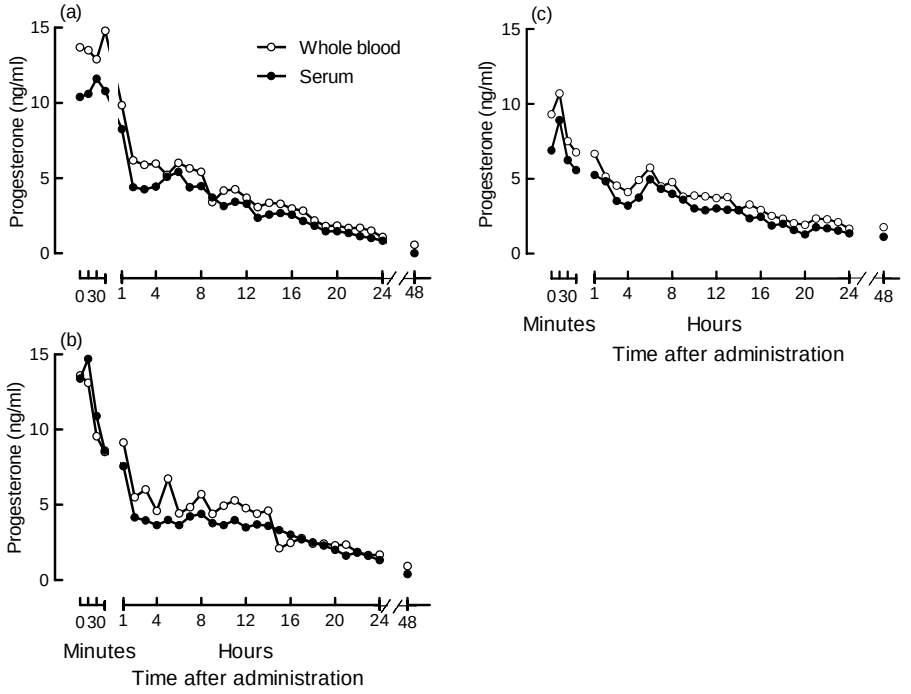

Figure 1.1.6 Changes in circulating progesterone (P) in whole blood (○) and serum samples (●) of three mares (a, b, c).

P concentrations were assayed by a chemiluminescent enzyme immunoassay after
administration of a single i.m. injection of prostaglandin F2α.

Section 2. Estradiol

1.2.1. Introduction

Estradiol (E) is secreted by the granulosa cells of the mature antral follicle in mares [22, 54, 58, 63, 64, 68]. It induces estrus and prepares the uterus for fertilization and implantation by promoting endometrial growth, development of the uterine gland, and secretion of uterine and vaginal mucus [54]. The physiological function of E is to induce embryo implantation in the uterus and maintain uterine conditions for the growing fetus during pregnancy [41, 74, 94–97]. Therefore, measurement of E is useful for detecting follicular and placental function in cyclic and pregnant mares. Circulating E has routinely been measured by RIA, EIA or FIA using plasma or serum samples extracted for E in equine clinics. Although these assays have sufficient sensitivity, accuracy, and simplicity, a period of one or two days is required to get their results. Therefore, there is a need for a rapid measurement technique that can be performed at equine clinics for early diagnosis.

The purpose of this study was to evaluate the validity of CLEIA system, as an accurate diagnostic tool for a rapid measurement of circulating E in mares using serum and whole blood samples without extraction in equine clinics.

1.2.2. Materials and Methods

Animals

129 thoroughbred mares (2-22 years of age) housed under natural conditions in Hokkaido, Japan were used for measuring the levels of circulating E.

Sample preparation and experiments

For the experiments determining the correlation between the results of CLEIA system and FIA, two pregnant mares, 4 and 15 years of age, were used. Serum samples were collected monthly from the jugular vein into commercially supplied plastic tubes at the day of copulation and the day of delivery (n = 27). The concentrations of E in all samples were measured by CLEIA and FIA.

For determining the correlation between the results for whole blood and serum, five pregnant mares, 8-19 years of age, were used. Whole blood samples were collected weekly from the jugular vein into heparin sodium-loaded tubes during day 233 of pregnancy and the day of delivery (n = 50). For serum sample collection, whole blood was drawn from the jugular vein of the same mares into plain blood collection tubes. Serum was separated by centrifugation at 1,700 ×*g* for 10 min. The concentrations of E in all samples were measured by CLEIA system.

For evaluating the pattern of circulating E during gestation in mares, 122 pregnant mares, ranging from 2-22 years of age, were used. Serum samples were collected during gestation from the jugular vein into commercially supplied plastic tubes. All mares delivered normally. The E concentrations in all samples were measured by CLEIA system. All procedures were carried out in accordance with the guidelines established by the institutional Animal Care and Use Committee of Tokyo University of Agriculture and Technology.

Hormone assay

1) CLEIA system

The concentrations of E in whole blood and serum samples were determined using the CLEIA system with the reagent for E (PF0171-K, LSI Medience Corporation) as described in section 1. The assay range of E by CLEIA system was between 20 and 2,000 pg/ml. The intra-assay coefficients of variance were 6.3% -12.9% for serum and 7.0%-12.4% for whole blood samples.

2) FIA

Serum concentrations of E were measured by using a time-resolved FIA kit (DELFIA, PerkinElmer, Waltham, MA, U.S.A.) as described previously [28, 48, 68] . Samples were added to a plate coated with an anti-rabbit IgG. After incubation and washing, a europium-labeled E and anti-E IgG was added and they were incubated. Enhancement solution was added and fluorescence was measured by a time-resolved fluorometer (ARVO X4, PerkinElmer, MA, U.S.A.). This system uses serum or plasma without extraction and the assay takes approximately 3 hr. The measurement was performed without extraction. The intra- and inter-assay coefficients of variation were 5.0% and 5.1%, respectively.

Statistics

Pearson's r and P value were calculated as described in section 1.

1.2.3. Results

The pattern of circulating E in the serum of two pregnant mares as measured by CLEIA system and FIA is shown in Fig. 1.2.1 as a sequential pattern throughout gestation. In Fig. 1.2.1a, serum concentrations of E measured by CLEIA system and FIA began to increase from 4 months after copulation, peaked at 8 months after copulation, and abruptly declined to the term. In Fig. 1.2.1b, the serum E concentrations measured by CLEIA system and FIA also began to increase from 5 months after copulation, peaked at 8 and 9 months after copulation followed by an abrupt decline to the term. In panel a, the values measured by CLEIA system tended to be high compared with those measured by FIA during the increasing phase between 4 and 8 months after copulation. This was also the same to panel b. The concentrations of E in serum samples as measured by CLEIA system were compared with those measured by FIA (Fig. 1.2.2). The results obtained by CLEIA system showed a significant correlation with those measured by FIA ($r = 0.9521$, $P < 0.0001$, $n = 27$).

The correlation of E concentrations in whole blood and serum samples from pregnant mares measured by CLEIA system is shown in Fig. 1.2.3. There was a significant positive correlation between the concentrations in whole blood and serum samples ($r = 0.9341$, $P < 0.0001$, $n = 50$).

To monitor physiological changes, the concentrations of E in serum samples obtained throughout the gestation period were measured by CLEIA system (Fig. 1.2.4). Circulating E began to increase in 15 weeks after copulation, and linearly increased until 31 weeks after copulation. High levels of serum E were maintained

between 30 and 43 weeks after copulation, followed by an abrupt decline (Fig. 1.2.4).

1.2.4. Discussion

The present study evaluated the validity of the rapid measurement of equine E using the new practical assay system, CLEIA system.

In the study of the method comparison of CLEIA system to FIA showed a positive correlation significantly ($r = 0.9521$, $P < 0.0001$, $n = 27$). In the present study, serum concentrations of E as measured by CLEIA system showed a parallel pattern to those measured by FIA, even though the values measured by CLEIA system tended to be higher than those measured by FIA during mid-gestation. Although the exact reason for this is not presently clear, the difference could be attributed to the differing cross reactivity of the anti-estradiol antibodies used in CLEIA system and FIA.

In the present study, E concentrations in whole blood and serum of mares showed excellent positive correlation, indicating that E concentrations can be measured in whole blood as well as in serum samples using CLEIA system.

In the present study, the physiological changes in circulating E levels during the gestation in mares were further examined using CLEIA system. The pattern of serum E levels during gestation in mares was similar to that observed in our previous results obtained using RIA [98, 99], indicating that CLEIA system correctly monitored the physiological changes in circulating E during the gestation.

Pregnancy with placentation is a peculiarity of mammals, including horses. Remarkable fetal growth occurs in the uterus during the late stages of pregnancy in all mammals. The most important factor includes the rapid elongation and cylindrical changes in the uterus to maintain fetal survival at the stage of rapid fetal growth during the late stages of pregnancy [41, 94–96]. P, E, prolactin and relaxin play key roles in the implantation of embryos, maintenance of pregnancy, and induction of parturition. Although the synergistic action of P and E is generally essential for mares, as in most mammals, for maintaining successful pregnancy, E plays a dominant role in the maintenance of uterine elongation when the fetus grows rapidly in late pregnancy [41, 94–96]. Therefore, monitoring circulating E and P is useful for detecting clinical signs of abortion, stillbirth, and perinatal fetal death late pregnancy in mares [48, 61, 68, 83].

E measurement is used to diagnose reproductive conditions at the equine clinic, and there is a need for a rapid measurement system in addition to the use of whole blood samples. In the present study, E concentrations in both whole blood and serum samples could be measured by CLEIA system within 30 min without ether extraction. Almost all the procedures were done automatically. This simple procedure makes hormonal evaluation by CLEIA system be an option for diagnosis of placental condition in pregnant mares in clinical practice. The only important point concerning the measurement of E by CLEIA system for use in the equine clinic is the detection limit of the assay. The basal level of circulating E during the estrous cycle [22, 58, 62, 63, 67] and the developmental stage [24, 49, 60, 66, 93] in mares is less than 20 pg/ml, and the detection limit of E by CLEIA system is 20

pg/ml. Extraction of E from serum by ether or other chemicals [64] will be required for measuring the basal E level in mares using this CLEIA system

In conclusion, CLEIA system would be useful in the equine hospitals as an accurate diagnostic tool for the rapid assay of E in pregnant mares [102].

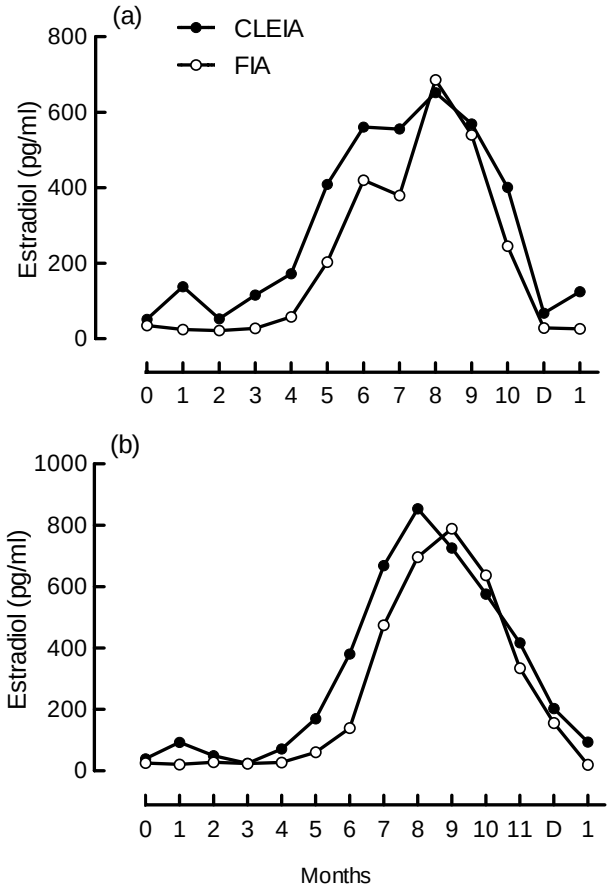

Figure 1.2.1 Serum estradiol (E) concentrations of two pregnant mares, as measured by chemiluminescent enzyme immunoassay (CLEIA) (●) and fluoroimmunoassay (FIA) (○).

E concentrations between last mating and 1 month after foaling were shown. "0" and "D" represent the days of copulation and delivery, respectively.

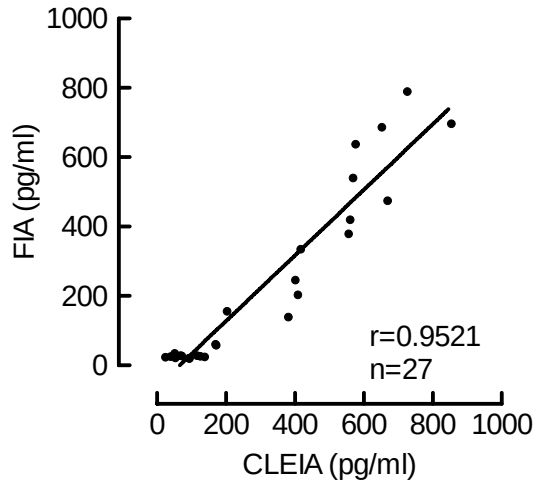

Figure 1.2.2 Correlation plots of estradiol (E) concentration in serum of pregnant mares measured by chemiluminescent enzyme immunoassay (CLEIA) and fluoroimmunoassay (FIA).

E concentrations measured by two methods were strongly correlated (r = 0.9521).

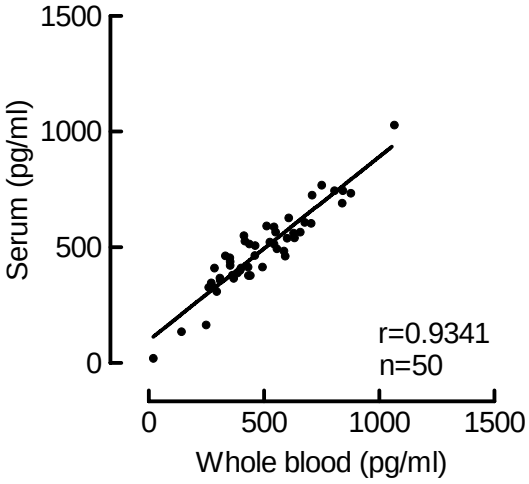

Figure 1.2.3 Correlation plots of estradiol (E) concentrations in the whole blood and serum of mares measured by chemiluminescent enzyme immunoassay.

E concentrations in whole blood and serum were strongly correlated (r = 0.9341).

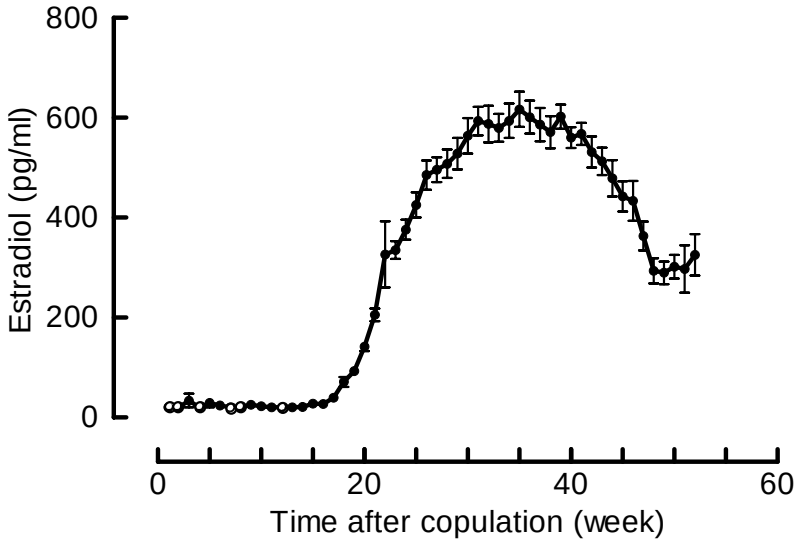

Figure 1.2.4 Changes in circulating estradiol during pregnancy of mares measured by chemiluminescent enzyme immunoassay system.

Results are expressed as means ± standard error of the mean (SEM) (n = 9-22). Values less than 20 pg/ml are described as white circles.

Section 3. Testosterone

1.3.1. Introduction

Testosterone (T), an androgenic hormone, plays important roles in various aspects of male reproduction including sexual behavior [55], spermatogenesis [26, 39] and construction of the blood-testis barrier [59]. Thus, T can be used as an indication of normal or abnormal reproductive function in males. Typically, T response to gonadotropin-releasing hormone (GnRH) or human chorionic gonadotropin (hCG) has been used for evaluation of fertility in many species including men [107], bulls [23], goat bucks [80], elephant bulls [86] and stallions [12, 72, 77, 78, 85]. While the GnRH challenge test enables evaluation of pituitary responsiveness in addition to testicular function by measuring luteinizing hormone (LH) or T concentrations, only testicular function can be assessed by the hCG challenge test. In clinical settings, the hCG challenge test seems a logical choice for evaluation of the testicular response of T secretion to the GnRH challenge test. The hCG challenge test can be performed in both breeding season and non-breeding season [76]. In stallions, for example, the hCG challenge test may be used to determine whether a stallion has cryptorchid testes (cryptorchidism) or has been castrated when testes are not found in the scrotum[4, 75].

Treatment with T products is also used in stallions with decreased libido [56]. When T products are used, monitoring of circulating T concentrations is strongly recommended to avoid overdosage of T products, which may result in testicular degeneration [10, 56].

In the present study, the usefulness of the CLEIA system for serum T assay in stallions was verified by comparing the results with T concentrations measured by a FIA, which has been used in stallions [49]. Then T measurement by the CLEIA system using whole blood as samples was verified for field use. Finally, the usefulness of the CLEIA system for evaluating testicular function in four typical cases found in the management of stallions was examined.

1.3.2. Materials and Methods

Animals

12 (No. 1 to 12) of various breeds in Hokkaido, Japan that were aged from 2 to 26 years were used in the present study (Tables 1.3.1, 1.3.2 and 1.3.3). They were kept in a pasture during the day and were housed individually in stalls at night or they were kept in individual stalls with a paddock. They were fed a maximum of 2 kg of concentrated feed per day with hay and water *ad libitum*.

Sample Collection

Blood samples were taken from the jugular vein into plain tubes for serum and into heparin sodium-loaded tubes for whole blood. The plain tubes were centrifuged at 1,700 x*g* for 10 min to separate serum, and serum samples were stored at -20°C until assay. The assay was performed on the same day as collection of whole blood. All procedures were carried out in accordance with the guidelines established by the institutional Animal Care and Use Committee of Tokyo University of Agriculture and Technology.

Hormone Assay

1) CLEIA System

Concentrations of T in serum and whole blood were measured by a CLEIA system using a reagent cartridge for the T assay (PF0191-K, LSI Medience Corporation) without extraction as described in section 1. The assay range of T by CLEIA was between 0.1 and 16.0 ng/ml. The intra-assay coefficients of variation were 8.9% at 0.87 ng/ml and 5.6% at 11.60 ng /ml for serum and they were 11.1% at 0.90 ng/ml and 4.2% at 14.60 ng/ml for whole blood according to the catalogue of the manufacturer.

2) FIA

Serum concentrations of T were measured by using a time-resolved FIA kit (DELFIA, PerkinElmer, Waltham, MA, U.S.A.) as described in section 2. The assay range of T by FIA was between 0.4 and 50.0 nmol/l (0.115 and 14.421 ng/ml). The intra- and inter-assay coefficients of variance were 6.0% and 12.9% at 1.20 ng/ml, 5.5% and 6.8% at 2.94 ng/ml and 5.6% and 5.6% at 7.95 ng/ml, respectively, according to the catalogue of the manufacturer.

3) RIA for luteinizing hormone (LH) concentrations

Serum concentrations of LH were determined by using RIA system as described previously [21]. 100 μl of standard in PBS (0.05M) containing 5% of BSA and sample were prepared. 50 μl of antiserum against equine LH (cat. no. AFP-

240580, provided by NIDDK. NIH, Bethesda, MD, U.S.A.) in PBS containing normal serum and EDTA (0.05M), and incubated at 4°C for 24 hr. After incubation, 50 µl of secondary antibody, anti-rabbit gamma globulin for LH diluted with PBS (0.05M) containing 5% polyethylene glycol, was added and incubated at 4°C for 24 hr and centrifuged at 1,700 ×g for 30 min at 4°C. After supernatant was removed and extra drops were swabbed, radioactivity was counted by gamma-counter. Highly purified equine LH (cat. no. AFP-5130B) was used for radioiodination and the reference standard. Intra-assay and inter-assay coefficients of variance were 12.6% and 15.1%, respectively.

Histological examination

The testes of the stallion 12 were examined histopathologically after orchiectomy in December 2014 (16 years of age). Orchiectomy was performed under general anesthesia that was induced with detomidine hydrochloride (Virbac, Milperra, Australia), diazepam (Takeda Pharmaceutical Company Limited, Osaka, Japan) and ketamine (Daiichi Sankyo Propharma, Tokyo, Japan) and maintained with isoflurane (Intervet, Tokyo, Japan).

A part of the testicular parenchyma was taken from the center of each testes. Samples of the testes were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. The paraffin-embedded testes were serially sectioned at 4 µm in thickness, and serial sections were treated with xylene and graded ethanol and then stained with hematoxylin and eosin and Masson trichrome stain. Testes of an adult Thoroughbred stallion that died at the age of 21 year due to

arteriorrhexis were used for a control.

For immunohistochemical (IHC) examination, testicular tissues were immune-stained against the equine LH receptor. Testicular paraffin sections were rinsed with 0.05% Triton-X 100 in phosphate buffered saline (PBS), and non-specific binding sites were blocked using 10% normal goat serum and 0.05% Tween 20 in PBS before incubation with a primary antibody. Epitope retrieval was carried out using a microwave (15 min in antigen retrieval citrate buffer, 10 mM sodium citrate, pH 6.0). After cooling down to room temperature, the sections were incubated overnight at 4°C with an anti-equine LH receptor antibody (rabbit polyclonal antibody, 1:20 dilution, Biorbyt, Cambridge, UK). The sections were rinsed in distilled water, followed by treatment with a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, U.S.A.) by the ABC method with a commercially available kit (Vector Laboratories). Immunostaining using a primary antibody was visualized using 3, 3'-diaminobenzidine tetrahydrochloride as a substrate (Sigma-Aldrich, St Louis, MO, U.S.A.) and counterstaining with hematoxylin.

Experimental Design

Study 1

To determine whether the CLEIA system can be used for stallion serum, serum T concentrations measured by the CLEIA system were compared with those measured by FIA. To create a broad range of circulating T concentrations, stallions 1 to 4 without any clinical signs were administered a single dose of 10,000 IU of

hCG (GESTORON 5000, Kyoritsu Seiyaku Corporation, Tokyo, Japan) intramuscularly (Table 1.3.1). Blood samples were taken immediately before and hourly until 6 hr after hCG administration and then daily for 7 days (n=56). Serum T concentrations were measured by the CLEIA system and FIA.

Study 2

To validate direct use of whole blood samples for the CLEIA system, T concentrations in whole blood and serum were determined with the CLEIA system. The system accommodates T concentrations in whole blood using hematocrit levels. In this study, the hematocrit level was fixed to 40%. Stallions 5 to 8 were administered T enanthate (Enarmon Depot, ASKA Pharmacoutical Co., Ltd, Tokyo, Japan) intramuscularly. Doses and protocols of administration and also blood sampling schedules are shown in Table 1.3.2 (n = 64). Stallions 7 and 8 were used twice with different protocols at an interval of 47 days.

Study 3

To evaluate the practical value of the CLEIA system, four stallions (Stallion 9 to 12) with reproductive abnormalities (Table 1.3.3) were subjected to hCG challenge tests.

Both testes of stallion 9 were smaller than normal size reported previously [100] when the stallion was introduced to the stud after retiring from racing. Stallion 10 had bilaterally enlarged testes compared with normal testicular size [100] and the testes were suspected to be tumors. Stallion 11 had no palpable testes

in the scrotum and no record of castration when the animal was introduced to our stud. When a mare at estrus came close, stallion 11 mounted and ejaculated as judged by the observation of tail flagging, but no spermatozoa was observed in the dismount semen. Stallion 12 had been used for mating and proven fertile in 2013. In 2014, at the age of 16, the number of sperm in the dismount semen was few and testicular atrophy was observed. Testicular size in stallion 12 was shown in Table 1.3.3. Stallion 9 was administered 5,000 IU of hCG intravenously [12] and stallions 10, 11 and 12 were administered 10,000 IU of hCG [85]. After administration of hCG, serum samples were obtained as in Study 1. Serum T concentrations were measured by the CLEIA system. In stallion 11, hCG challenge tests were performed twice at an interval of two months. Stallion 12 was subjected to hCG challenge test in 2010 for hCG trial in fertile stallions and T concentrations were measured by RIA. To evaluate the endocrine function of the hypothalamus-pituitary-gonadal axis, basal concentrations of LH and T in stored serum were measured in stallion 12 during the breeding and non-breeding seasons in 2014 (16 years of age) and in 2009 to 2013 (11-15 years of age). Blood samples were collected from the jugular vein of stallion 12 during the breeding season (n = 4, April to July in 2014) and during the non-breeding season (n = 3, September to November in 2014). Blood samples were also collected from stallion 12 before the development of testicular atrophy in the breeding season (n = 5, April, 2009 to 2013) and in the non-breeding season (n = 5, October, 2009 to 2013). Blood samples were also collected from apparently normal Thoroughbred stallions from 14 to 16 years of age during the breeding season (n = 6, April) and the non-breeding season (n = 6,

October) as controls. Serum concentrations of LH were determined by RIA and serum concentrations of T were measured by a CLEIA system.

Statistics

In Studies 1 and 2, Pearson's correlation (r) was calculated as described in section 1. In Study 1, Dunnett's multiple comparison test was performed for comparison of T concentrations after administration of hCG with those before administration by using GraphPad Prism 5 for Windows. In study 3, One-way ANOVA and Tukey's multiple comparison test were performed to detect significant changes in the concentrations of LH and T of stallion 12 using GraphPad Prism version 5.

1.3.3. Results

Study 1

Serum T concentrations measured by the CLEIA system and FIA showed a strong correlation ($r = 0.9865$, $P < 0.01$, $n = 56$, Fig. 1.3.1). Changes in serum T concentrations after hCG administration measured by the CLEIA system and FIA were similar (Fig. 1.3.2). Serum T concentrations showed bimodal peak. The first peak appeared within 3 hr after administration of hCG, and the second one appeared 3 or 4 days after administration of hCG. T concentrations between 2 and 5 days after administration of hCG were significantly higher than those before

administration.

Study 2

Whole blood T concentrations and serum T concentrations measured by the CLEIA system showed a strong correlation ($r = 0.9665$, $P < 0.01$, $n = 64$, Fig. 1.3.3). Changes in T concentrations in whole blood after T product administration in each stallion are shown in Fig. 1.3.4. T concentrations increased after single administration of 100 mg or 250 mg or double administrations of 100 mg T enanthate; however, the increase was not clear. T concentrations after double or quarterly administrations of 250 mg T enanthate clearly increased.

Study 3

In stallion 9, serum T concentrations showed bimodal changes similar to those in normal stallions 1 to 4 (Figs. 1.3.2 and 1.3.5a). Stallion 10 showed no obvious increase in serum T concentration during the seven-day study period after hCG administration (Fig. 1.3.5b). In stallion 11, serum T concentration showed no increase for the first 6 hr but increased after 24 hr. Serum T concentrations after the second administration tended to be lower than those after the first administration (Fig. 1.3.5c). Stallion 12 had subjected to hCG challenge test four years before the present study (12 years of age) and had shown an increase in T indicating a normal testicular function. However, an increase in the serum concentration of T was not observed after intravenous administration of hCG in the present study (Fig. 1.3.5d). In breeding season, serum concentrations of LH of

stallion 12 in 2014 (16 years of age), after testicular atrophy had developed, were significantly higher than those in 2009 to 2013 (11-15 years of age), before testicular atrophy had developed and controls. In non-breeding season, serum concentrations of LH of stallion 12 in 2014 were also significantly higher than those of stallion 12 from 2009 to 2013 and controls (Fig. 1.3.6a). Although serum concentrations of T in breeding season tended to be higher than those in non-breeding season, there was no significant difference among the groups (Fig. 1.3.6b). The stallion was retired from mating and castrated. The testes were examined histologically. It was revealed that there were seminal tubules with arrested spermatogenesis and sertoli cells within these seminal tubules had large empty intracytoplasmic vacuoles (Fig. 1.3.7b, d). The amount of collagen fibers was increased (Fig. 1.3.7f). Leydig cells in the testes were swollen (Fig. 1.3.7d) and LH receptors were not detected by IHC in Leydig cells in the testes of stallion 12 (Fig. 1.3.7h).

1.3.4. Discussion

The present study demonstrated that the CLEIA system can be used for measurements of T concentrations in stallions. First, serum T concentrations measured by the CLEIA system were compared with those measured by FIA, an established assay system for stallion serum T measurement [49]. T concentrations measured by the CLEIA system were almost the same as those measured by FIA ($r = 0.9867$). Furthermore, the secretion pattern of T was very similar to that reported previously [12]. These results together with the rapidity of the CLEIA

system indicated that the system is a valuable and practical system for measuring T concentrations in stallions as an alternative to FIA. T concentrations can be measured in a much shorter time by the CLEIA system than by FIA (30 min vs 3 hr). Furthermore, the CLEIA system allows us to obtain results with a single step of sample application on a reagent cartridge, while the FIA system requires complex and multiple manipulations.

T concentrations in whole blood measured by the CLEIA system were almost the same as those in serum ($r = 0.9665$). This system accommodates T concentrations in whole blood by using hematocrit levels. In a previous study conducted in human subjects, T concentrations in whole blood were found to be strongly correlated with those in serum when hematocrit levels were fixed at 40% [18]. In the present study, hematocrit levels were also fixed at 40% according to that previous study, and T concentrations in whole blood were found to be highly correlated with those in serum in stallions. The rapidity and compatibility of whole blood as samples for the CLEIA system may make the system a useful tool and enable better T therapy for decreased libido. It is difficult to determine the dose and timing of T administration to achieve effective blood T levels and to avoid overdosing. In this study, a single administration of 100 or 250 mg and double administrations of 100 mg of T enanthate did not result in high T concentrations in circulation. Administration of 250 mg T enanthate for more than 2 days resulted in an increase in T concentrations for several days. Administration of T enanthate is used for stallions with decreased libido; however, it has been shown that administration of an excess amount of T impaired spermatogenesis and decreased

the testicular volume in stallions [10]. Administration of T enanthate is an option for treatment of low libido in my experience. Previously, one stallion in my stud with low libido was able to mount with an increase of T concentrations by administration of T enathate. However, blood T levels to be attained for restoring libido are unknown, and an appropriate T administration protocol has not been established [56]. Obviously, further research is needed, and the CLEIA system used in the present study would be a useful tool.

Measurement of T concentrations is often needed in a stud. The risk of reproductive abnormalities, such as testicular degeneration and testicular tumor, increases with aging [87, 106]. When stallions are moved from one farm to another, their clinical records sometimes become uncertain. If a stallion shows an absence of testes in the scrotum, it must be determined whether the stallion was castrated or whether the stallion has cryptorchid testes. In a Thoroughbred stud, some stallions would have been retired from racing at the end of the year, and the period between retirement from racing and the start of breeding would be only 2 to 3 months. Stallions are trained to mate with mares during this short period. Some stallions may take a while to start mounting mares and may have low libido. Although the frequency may be low, some new young stallions retiring from racing have small testes compared with testes in stallions of equivalent age. In such cases, veterinarians in the stud must evaluate the testicular function of these stallions. Measurement of T concentration with or without hCG challenge may be useful for distinguishing cases of cryptorchidism and castration, and hCG challenge is also a useful method for distinguishing infertile and normal stallions. In this study, four

stallions with suspected reproductive abnormalities were subjected to the hCG challenge test, and the response of T concentrations was determined by using the CLEIA system.

Stallion 9 with small testes showed a T response to hCG that was similar to the response found in normal stallions in Study 1 (Fig. 1.3.2). The observed T response suggested normal reproductive ability of stallion 9. The conception rate of stallion 9 in the first breeding season was more than 70%, indicating that the testes of stallion 9 were small because of individual difference and that spermatogenesis in stallion 9 was normal.

In stallion 10 with enlarged testes, T concentrations did not increase after hCG administration, indicating the possibility that Leydig cells had lost their steroidogenic capacity. The stallion died one year and four months after the hCG challenge test. The testes were examined histologically and a diagnosis of seminoma was made. Seminoma generally does not affect T production [30]. Testicular sizes when stallion 10 died were larger (left: 24.0 x 17.0 x 11.0 cm, right: 26.0 x 14.5 x 9.0 cm) than the sizes when the hCG challenge test was performed. It is no clear whether seminoma influenced the ability of T secretion.

In stallion 11, an increase in T concentrations after hCG administrations indicated the presence of testes [4, 75]; thus, stallion 11 was diagnosed as having cryptorchid testes, not having been castrated. Stallion 11 was castrated by using laparoscopy to prevent cryptorchidism from developing into tumors [50, 88]. Both testes were found in the abdominal cavity. T concentrations after the second administration tended to be lower than those after the first administration. It is

unlikely that antibodies against hCG were produced after a single administration of hCG [79]. It was not clear why T concentrations after the second experiment were lower than those after the first experiment in this study.

In stallion 12 in 2010 (12 years of age), following administration of hCG, serum concentrations of T increased and reached the first peak at 4 hr, and declined gradually. The serum concentrations of T began to increase again from the second day and reached the second peak 3 days after hCG administration, followed by a gradual decline by 7th days after hCG administration. On the other hand, an increase in the serum concentrations of T was not observed after intravenous administration of hCG in stallion 12 in this study in 2014 after development of testicular atrophy (Fig. 1.3.5d), indicating that Leydig cells in the testes of stallion 12 lost reactivity to hCG due to loss of LH receptors in Leydig cells. Our previous studies demonstrated that there were clear seasonal changes in circulating LH and T in Thoroughbred stallions [25, 65]. The highest and lowest circulating concentrations of both LH and T were observed in the breeding and non-breeding seasons, respectively. Basal T concentrations in stallion 12 in 2014 were not different from T concentrations in stallion 12 before 2013 and controls. In the meantime, LH concentrations in stallion 12 in 2014 were significantly higher than those in stallion 12 before 2013 and controls. Although LH concentrations were affected by season, LH concentrations of stallion 12 in non-breeding season in 2014 remained high compared with those in other groups. This result was consistent with the loss of LH receptors in Leydig cells [104].

In these clinical cases, T measurement by the CLEIA system was useful for

evaluation of testicular function and for judging the existence of normal testicular tissue.

The minimum detection level of the CLEIA system for human is 0.1 ng/ml. It may not be sensitive enough to measure basal T concentration at rest in a stallion which is as low as 300 and 402 pg/ml in the non-breeding and breeding seasons, respectively [42]. Therefore, a sample may need to be concentrated to ten times, for example, to determine the actual blood concentration, although that may jeopardize the rapidity and simplicity of the system.

In conclusion, it was shown that the CLEIA system can be used for measuring T concentrations in both serum and whole blood of stallions. By using the CLEIA system, it is possible to measure T concentrations rapidly and easily. The system is useful for diagnosis of reproductive abnormalites by an hCG or GnRH challenge test and for treatment of stallions with low libido by administration of a T product in the Thoroughbred industry [103].

Stallion No.	Breed	Age	hCG Dosage (IU)	Clinical findings	Date
1	Warmblood	2	10,000	No clinical symptom	2010/9/23
2	Warmblood	2	10,000	No clinical symptom	2010/9/23
3	Warmblood	2	10,000	No clinical symptom	2010/10/4
4	Halfbred	3	10,000	No clinical symptom	2010/10/7

Table 1.3.1 Breed, age, clinical signs, and hCG challenge protocol of stallions in Study 1.

Table 1.3.2 Breed, age, and T administrations protocol of stallions in Study 2.

Stallion No.	Breed	Age	Dosage per treatment (mg)	No. of days for daily treatments	Period of daily blood collection	Date of T administration
5	Halfbred	7	100	1	Day 0 to 7	2013/1/7
6	Warmblood	4	100	2	Day 0 to 7	2013/1/7
7(1st)	Warmblood	4	250	1	Day 0 to 8	2013/1/7
7(2nd[a])			250	4	Day 0 to 14	2013/2/23
8(1st)	Halfbred	6	250	2	Day 0 to 8	2013/1/7
8(2nd[a])			250	4	Day 0 to 14	2013/2/23

[a] The second T enanthate treatment for both stallions 7 and 8 were started 47 days after the first day of the first treatment.

Stallion No.	Breed	Age	hCG Dosage (IU)	Clinical findings	Date
9	Thoroughbred	9	5,000	Small size testes, left: 6.4 x 2.9 x 2.7, right: 7.6 x 4.1 x 2.7[a]	2011/1/8
10	Haflinger	26	10,000	Enlarged testes suspected tumor, left: 19.4 x 13.0 x 10.2, right: 20.8 x 14.0 x 12.0[a]	2013/1/29
11[b]	Warmblood	3	10,000	Absence of testes in the scrotal, suspected cryptorchidism or castrated	2015/9/4, 2015/11/17
12[c]	Thoroughbred	16	10,000	Testicular atrophy, left: 6.7 x 3.4 x 4.5, right: 6.2 x 3.7 x 2.5[a]	2014/2/16

Table 1.3.3 Breed, age, clinical signs and hCG challenge protocol for stallions in Study 3.

[a] Testicular sizes are indicated as length x height x width (cm).
[b] Stallion 11 was administered 10,000 IU of hCG twice with an interval of two months.
[c] Stallion 12 was performed hCG challenge test in 2010, before development of testicular atrophy. At that time, T concentrations were measured by RIA.

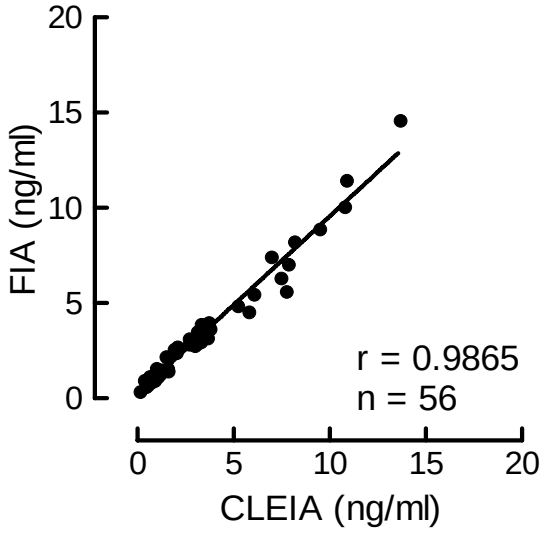

Fig. 1.3.1 Correlation of testosterone (T) concentrations in serum measured by the chemiluminescent enzyme immunoassay (CLEIA) system and by fluoroimmunoassay (FIA).

Stallions 1 to 4 were administered 10,000 IU of human chorionic gonadotrophin (hCG) intramuscularly, and T concentrations in blood samples (n = 56) were measured by the CLEIA system and FIA. T concentrations measured by the two methods were strongly correlated (r = 0.9865).

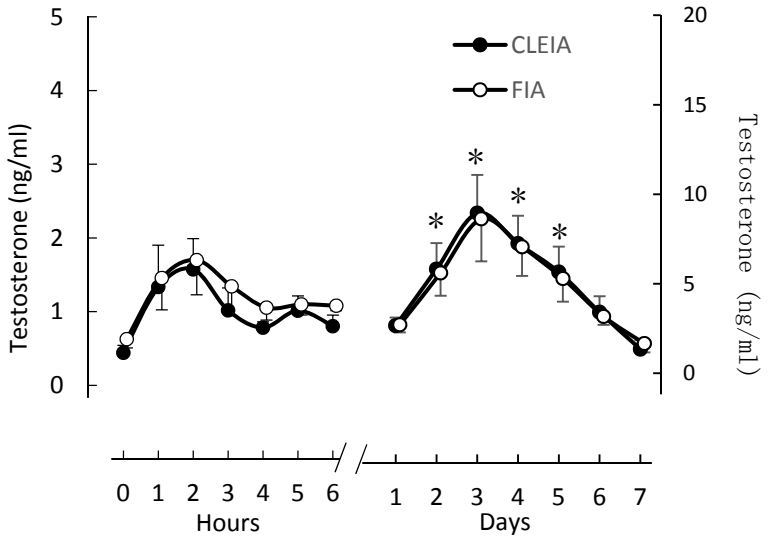

Fig. 1.3.2 Comparison of testosterone (T) concentrations measured by the chemiluminescent enzyme immunoassay (CLEIA) system and by fluoroimmunoassay (FIA).

Four stallions (1 to 4) with no clinical signs were administered 10,000 IU of human chorionic gonadotrophin (hCG). T concentrations in serum samples were measured by the CLEIA system and FIA. T concentrations are shown as averages ± SEM. The left vertical axis shows hourly data and the right vertical axis shows daily data. An asterisk indicates that the mean of T concentrations is significantly higher than that before administration.

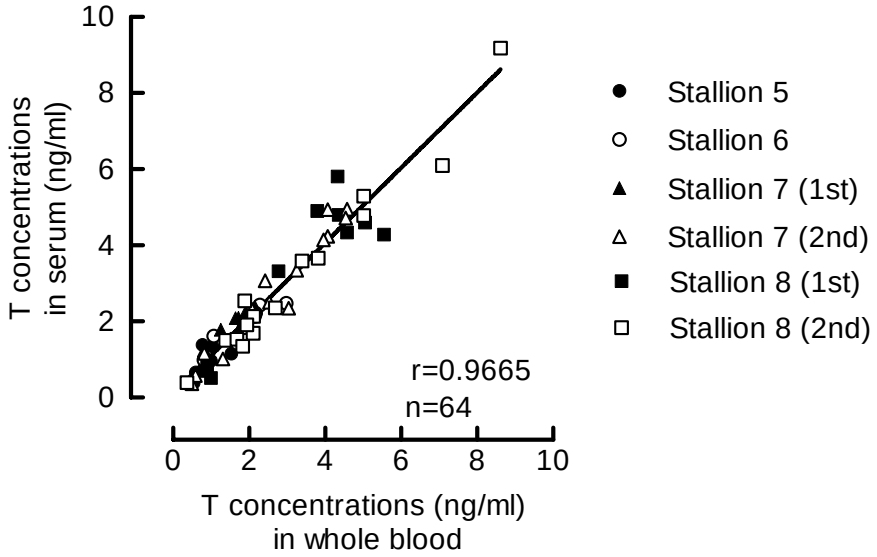

Fig. 1.3.3 Correlation of testosterone (T) concentrations in whole blood and serum measured by the chemiluminescent enzyme immunoassay (CLEIA) system.

Stallions 5 to 8 were administered (i. m.) T enanthate (100 or 250 mg) for 1 to 4 days (daily). Blood samples (n = 64 in total) were collected before and after T enanthate administration for 5 to 8 consecutive days. See Table 1.3.2 for details of the T enanthate treatment protocol in each stallion.

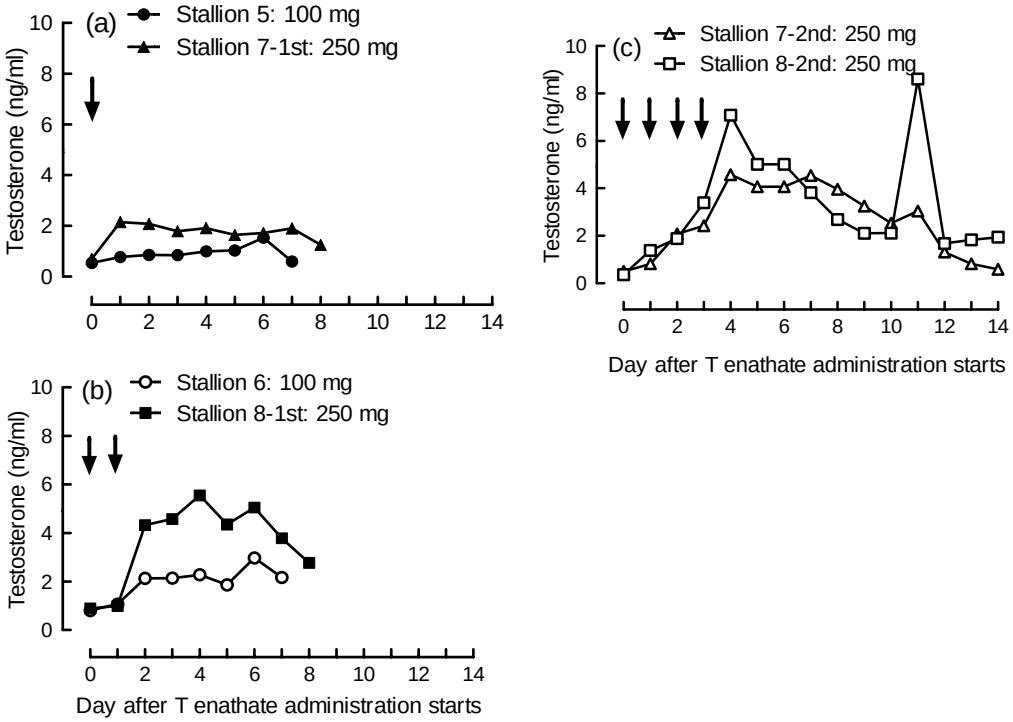

Fig. 1.3.4 Concentrations of testosterone (T) in whole blood after T enathate administration.

Stallions 5 to 8 were administered T enathate as shown in Table 1.3.2. Arrows indicate administration of T enathate.

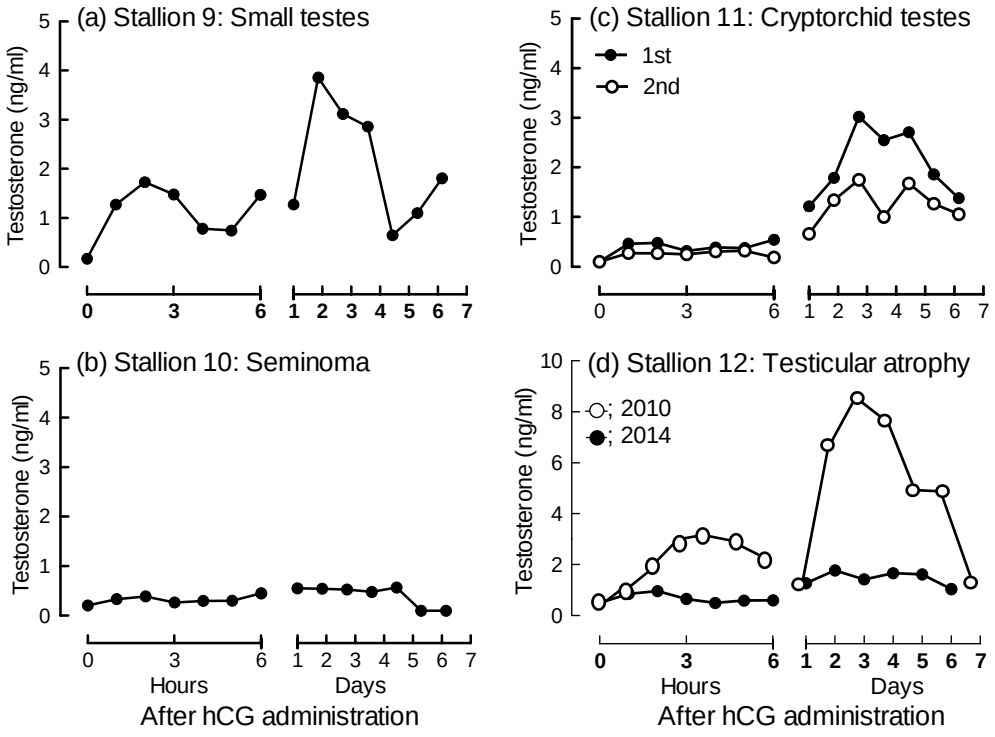

Fig. 1.3.5 Changes in serum testosterone (T) concentrations after human chorionic gonadotropin (hCG) administration in stallions with reproductive abnormalities.

Stallion 9 (5,000 IU) and stallions 10, 11 and 12 (10,000 IU) were administered hCG to determine T responses to hCG administration. See Table 1.3.3 for clinical findings of each animal. Stallion 11 was administered hCG twice with a two-month interval. Stallion 12 was performed hCG challenge test in 2010. T concentrations were measured by radioimmunoassay at that time.

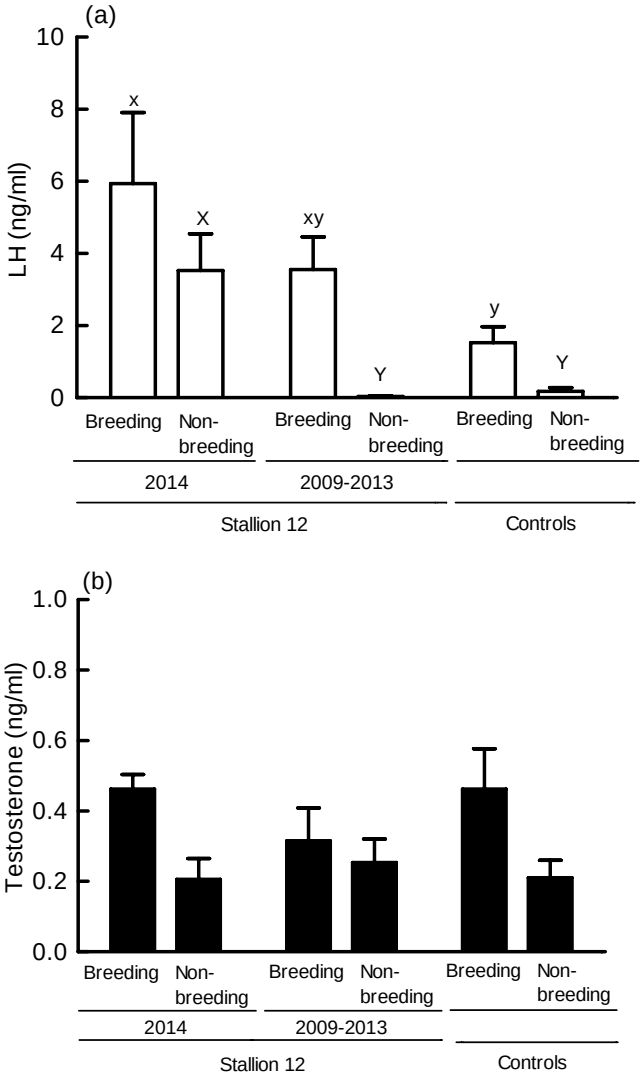

Figure 1.3.6 Basal concentrations of circulating luteinizing hormone (LH) (a) and testosterone (b) in a Thoroughbred stallion (stallion 12) during the breeding (April to July) and non-breeding seasons (September to November) and in the intact adult Thoroughbred stallion.

Stallion 12 in 2014 after the development of testicular atrophy in the breeding season (n = 4). Stallion 12 in 2014 after development of testicular atrophy in the non-breeding season (n = 3). Stallion 12 in 2009-2013 before the development of testicular atrophy in the breeding season (n = 5). Stallion 12 in 2009-2013 before the development of testicular atrophy in the non-breeding season (n = 5). Intact Thoroughbred stallions in the breeding season (n = 6). Intact Thoroughbred stallions in the non-breeding season (n = 6). Results are expressed as means ± SEM. x, y or X, Y: means are significantly different within the same season ($P < 0.05$).

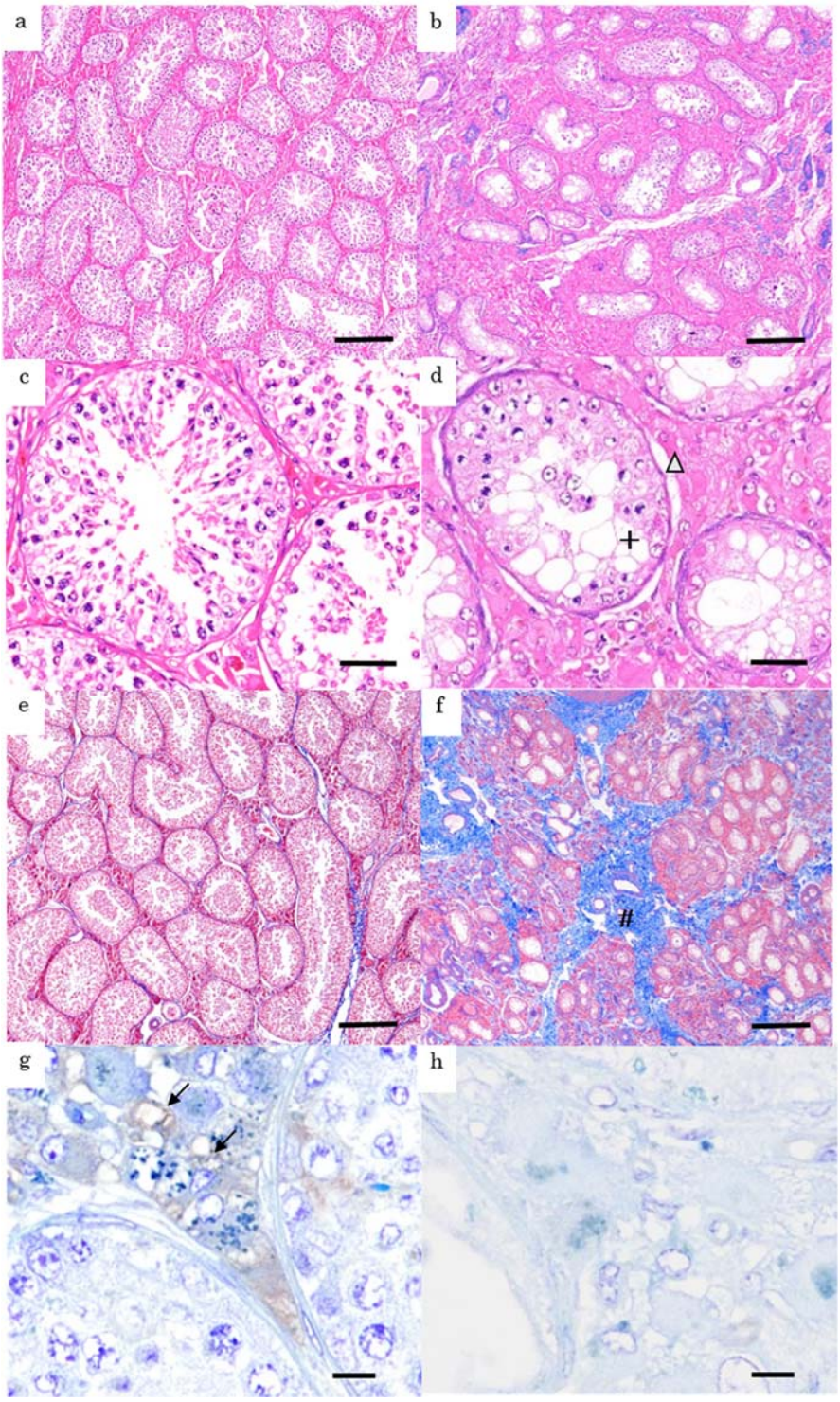

Figure 1.3.7 Histological (a-f) and immunohistochemical (g, h) observations of the testes of an intact Thoroughbred stallion (a, c, e, g) and Thoroughbred stallion 12 (b, d, f, h).

Complete spermatogenesis was observed within the lumen of the intact adult Thoroughbred stallion (a, c). Spermatogenesis was arrested in stallion 12 (b,

d). Sertoli cells within tubules had large empty intracytoplasmic vacuoles in stallion 12 (d, +). Leydig cells (d, white arrowhead) were swollen. The amount of collagen fibers was increased (f, #). In immunohistochemical sections, LH receptors were observed in Leydig cells in the testes of an intact adult Thoroughbred stallion (g, black arrow) but not in Leydig cells in the testes of stallion 12 (h). a-d: Hematoxylin and eosin stain. e, f: Masson trichrome stain. g, h: immune-stain against the equine LH receptor. Bars in panels a, b, e and f represent 200 μm, bars in panels c and d represent 40 μm, and bars in panels g and h represent 10 μm.

Summary

In order to evaluate the validity of chemiluminescent enzyme immunoassay (CLEIA) system for measurement of progesterone (P), estradiol (E) and testosterone (T) concentrations in horses, concentrations of P, E and T measured by CLEIA system were compared with those measured by radioimmunoassay (RIA), enzyme immunoassay (EIA) or fluoroimmunoassay (FIA). CLEIA system showed significant correlations with those assays and it is confirmed that CLEIA system can be used for evaluation the concentrations of P, E and T in horses. CLEIA system for measurement of P concentrations in mares are useful for evaluation of corpus luteum. It is helpful to distinguish mares in diestrus from those in estrus at the mating in stud. CLEIA system for measurement of E concentrations in pregnant mares are useful for evaluation the placental function. However, it cannot use for non-pregnant mares because E concentrations in non-pregnant mares are lower than the assay limit of CLEIA system, 20 pg/ml. CLEIA system for measurement of T concentrations in stallions is useful for evaluation of testicular function after human chorionic gonadotrophin (hCG) challenge test, diagnosis for cryptorchidism and monitoring the fluctuation of T concentrations after T product administration.

Hormonal concentrations of P, E and T in whole blood samples measured by CLEIA system and compared with those measured in serum samples by CLEIA system. Concentrations of P, E and T in whole blood showed a strong correlation with those in serum samples. Whole blood samples can be used as serum samples

for measurement by CLEIA system. It becomes possible to get result of hormonal concentrations rapidly by using whole blood samples measured by CLEIA system.

CHAPTER II Management of equine coital exanthema presenting serious problems in stud

Section 1. Clinical cases of equine coital exanthema and trial of treatment with valacyclovir

2.1.1. Introduction

Equine coital exanthema (ECE) is caused by equine herpesvirus 3 (EHV-3), which is a member of the *Varicellovirus* genus, subfamily *Alphaherpesvirinae*.

EHV-3 causes latent infection as do other herpesvirus [7–9]. The prevalence of the virus in Japan is unknown. Equine herpesvirus 1 and 4 are also members of the *Varicellovirus* genus, subfamily *Alphaherpesvirinae*. These are cause of equine rhinopneumonitis and abortion. Equine rhinopneumonitis is a notifiable-infectious disease, but ECE is not. EHV-3 is transmitted via both direct contact and indirect contact. It is also transmitted by coitus and artificial insemination [2, 6, 9, 29]. Infection with the virus results in the development of papules, vesicles, pustules and ulcers on the vaginal area in mares and on the penis and prepuce in stallions [2]. These regional symptoms are cured in two to three weeks [2, 7, 29, 35, 81]. Treatments for the disease are very limited. In Japan, firing the affected area with silver nitrate has been used on a regular basis. An antibiotic is used for prevention of complications [29, 81]. Some researchers have investigated the effectiveness of acyclovir, an anti-herpesvirus agent [2, 6, 19, 81]; however, the effectiveness of valacyclovir, a prodrug of acyclovir, has not been reported.

The occurrence of ECE has been reported in many countries [6, 47, 81]. In Japan, symptoms suggesting ECE have been reported, but isolation of EHV-3 was reported in only one case. It was isolated from draft mares in Iwate Prefecture in 2004 [81].

As reported here, two stallions developed symptoms suggestive of ECE, and EHV-3 was isolated from both stallions. Valacyclovir and acyclovir, anti-herpesvirus agents, were used for treatment, and it was investigated whether valacyclovir is effective for treating ECE.

2.1.2. Materials and Methods

Cell culture and virus

Fetal horse kidney (FHK) cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and used for virus isolation and serum neutralization (SN) tests. For the maintenance medium, the serum concentration was reduced to 4%. The Iwate-1 strain was propagated in FHK cells and used as the reference strain of EHV-3 in this study [81].

Samples from affected horses

Stallion A and stallion B developed ECE clinically on April 10, 2015 and May 14, 2015, respectively. The day after the onset, swabs were obtained from penile lesions of both stallions and placed in 1 ml of MEM supplemented with 50 μg/ml of gentamycin. The eluted samples were centrifuged at 670 ×*g* for 10 min at 4°C to pellet debris, and the supernatant fluids were used for virus isolation.

Virus isolation

100 μl of each of the supernatant fluids of swab samples was inoculated onto FHK cells cultured in a 6-well plate (Thermo Fisher Scientific, Waltham, MA, U.S.A.). After adsorption at 37°C for 30 min under a 5% CO_2 atmosphere, the cells were washed twice with MEM and the maintenance medium was added. Then the cells were cultured at 37°C under a 5% CO_2 atmosphere.

Serum samples

Sera from stallions A and B were collected on April 11, April 27, May 27, June 30, July 27 and August 31 in 2015, and on May 15, June 3 and September 16 in 2015, respectively.

SN test

Antibodies against EHV-3 in sera were determined by the SN test using the EHV-3 isolate from the stallion A and FHK cells as described previously [81]. Serial two fold dilutions, beginning with a 1:2 dilution, of the serum were mixed with an equal amount of the EHV-3 isolate containing a 200 median tissue culture infectious dose per 0.1 ml before incubation at 37°C for 60 min. One well of cell cultures was inoculated with 0.1 ml of each virus-sample mixture and incubated at 37°C. The results were read 7 days postinoculation. SN titer was expressed as the reciprocal of the highest dilution that completely inhibited CPE.

Plaque reduction assays

Antiviral activity of valacyclovir (Sigma-Aldrich, St. Louis, MO, U.S.A.) and that of acyclovir (Sigma-Aldrich) against EHV-3 were determined. FHK cells were seeded in 6-well culture plates and were cultured in a growth medium (MEM supplemented with 10% FCS) at 37 °C for 24 hr. Then the growth medium was removed and FHK cell monolayers were cultured with various concentrations of each compound, from 1 mg/ml to 250 ng/ml in MEM supplemented with 4% FCS at 37°C for 24 hr. The culture medium was removed again and FHK cells were infected with 50 to 100 plaque-forming units of EHV-3/well. After 1-hr adsorption,

HFK cells were washed three times with MEM and overlaid with MEM containing the same concentration of each compound, 4% FCS and 0.9% Agar Noble (Difco, Detroit, MI, U.S.A). Plaques were counted two days after infection. The 50% effective concentration (EC_{50}) for reduction in plaque number was calculated from dose-response curves generated from the data.

2.1.3. Results

Case 1

Stallion A was a 14-year-old Thoroughbred. The stallion mated with two or three mares every day from mid of February until the occurrence of ECE. On April 10, 2015, some papules were found on the penis, but this stallion mated with three mares on the same day (date of occurrence). The next day, the papules were ruptured and exudative fluid was observed. The stallion showed signs of pain by palpation of the penis, and mating was impossible. After EHV-3 was isolated from a penile swab [46], oral administration of valacyclovir (Valtrex, GlaxoSmithKline K. K., Tokyo), which is a prodrug of acyclovir and an anti-herpesvirus agent, was started (27 mg/kg/8 hr for 2 days, 18 mg/kg/12 hr for 8 days) [53] from the 2nd day of occurrence. Washing the affected area with hypochlorous acid solution (50 ppm, pH 6.5) and application of 1% nadifloxacin ointment were started on the same day. On the 3rd day, body temperature rose and appetite was lost. Flunixin meglumine was therefore administered (1.1 mg/kg). The affected area did not expand after starting administration of valacyclovir and gradually dried from the 5th day. Local treatment for the penis was stopped on the 8th day, and oral administration of

valacyclovir was stopped on the 11th day. Symptoms of the penile area are shown in Fig. 2.1.1a. The stallion started mating again on the 18th day, and the stallion had no problems with mating. The serum antibody titers are shown in Table 2.1.1. One of the three mares the stallion mated with on the date of occurrence developed papules 5 days after mating, and EHV-3 was isolated from a vaginal swab.

Case 2

Stallion B was a 13-year-old Thoroughbred. The stallion mated with one or two mares almost every day. On May 14, 2015, some papules had developed on the stallion's penis and the number of papules gradually increased (date of occurrence). Some of the papules ruptured and exudate was observed. Because the symptoms were similar with those observed in stallion A, the stallion was administered valacyclovir orally at the same dose as that for stallion A on the date of occurrence before the isolation of EHV-3. In the results, EHV-3 was isolated from a penile swab [46]. For local treatment, the penis was washed with hypochlorous acid solution and 5% acyclovir ointment, an anti-herpesvirus agent, was applied from the 2nd day. The affected area did not expand from the 2nd day and the ruptured pustules gradually became scarred. Local treatment was stopped on the 7th day and oral administration of valacyclovir was stopped on the 10th day. Symptoms of the penile area in stallion B are shown Fig. 2.1.1b. The stallion started mating on the 19th day, and the stallion had no problems with mating. The serum antibody titers are shown in table 2.1.1.

Antiviral activity of valacyclovir

The dose-response curves are shown in Fig. 2.1.2. The EC_{50} values are 3 μg/ml for valacyclovir and 2.3 μg/ml for acyclovir. Both valacyclovir and acyclovir were effective for inhibiting replication of EHV-3.

2.1.4. Discussion

Symptoms suggestive for ECE have often been observed in Japan, but the exact causes were not investigated in most cases. Isolation of EHV-3 was reported previously in only one case in Japan and it was isolated from mares [81]. This is the first report of isolation of EHV-3 from stallions in Japan. The two stallions from which EHV-3 was isolated were kept at different studs. The distance between the two studs is more than 70 km, and the stallion handlers did not pass between the two studs in the breeding season. There was no mare that was mated in both of the stallions. Thus, it is possible that EHV-3 was prevalent subclinically among Thoroughbreds in Japan.

During the occurrence of ECE, the stallions had to stop mating because there was pain by palpation of the penis and because EHV-3 is transmitted by coitus. Both of the stallions stopped mating for more than two weeks. Because their fees for mating were very high, financial losses were enormous and owners who planned to mate their mares with stallion A or B had to change their mating plan. ECE therefore has huge negative effects on the Thoroughbred racing horse industry.

Stallions are at high risk for exposure to EHV-3 in the breeding season

because they are in contact with many mares, and popular stallions are very busy and under a lot of stress in the breeding season [6]. According to some reports, stress causes re-activation of the virus latently in infected horses [2, 9, 51].

Approaches to therapy for ECE are very limited. In Japan, firing the affected area with silver nitrate has been used, but this is painful and its effect is unknown. Some researchers have used acyclovir for treatment of ECE. In a previous study, a stallion and some mares with ECE responded well to application of acyclovir, and an inhibitory effect of acyclovir on EHV-3 was shown by plaque reduction assays [19]. Some researchers have used valacyclovir, a prodrug of acyclovir, for treatment of EHV-1 infection but not for treatment of EHV-3 infection [31–33, 53]. Some studies showed that valacyclovir maintained an effective serum concentration for a longer time than did acyclovir [31, 33, 53]. We therefore expected that valacyclovir would be more effective than acyclovir for treatment of ECE. We used valacyclovir for systemic treatment, and this is the first study in which valacyclovir was used orally for treatment of ECE. However, it took more than 2 weeks for both stallions to start mating again, and this period is almost the same as that when ECE was treated without an anti-herpesvirus agent [2, 7, 29, 35, 81]. These results suggest that once papules have ruptured, it takes more than 2 weeks for regeneration of the penile area. Treatment with valacyclovir did not greatly shorten the duration of disease. Garré et al. [32], reported that unbound plasma acyclovir concentrations were maintained between 1.7 μg/ml and 3.0 μg /ml after valacyclovir administration (40 mg/kg body, 3 times daily) in ponies. The isolate used in this study is susceptible to valacyclovir *in vitro* at a concentration

of 3.0 μg/ml. Therefore, in this study, the dosage regimen consisting of 27 mg/kg/8 hr for 2 days and 18 mg/kg/12 hr for 8 days might have resulted in a plasma concentration that did not exceed the EC_{50} value of EHV-3. However, the affected area did not expand after oral administration of valacyclovir, suggesting the potential of valacyclovir for suppression of EHV-3 activity clinically.

Stallion A was not a subclinical case and EHV-3 was transmitted from other mares because the stallion's antibody titer had never increased for 8 years before occurrence. On the other hand, the antibody titer of stallion B before occurrence was not investigated, and whether the cause of ECE symptoms was re-activation or transmission from mares was unknown. Serum antibody titers did not increase immediately after occurrence in contrast to the immediate increase in antibody titers in previous studies [7, 81], but they increased 2 months and 4 months after occurrences in stallion A and stallion B. Oral administration of valacyclovir might suppress the increase in antibody titers.

It has been reported that EHV-3 was transmitted not only from horses with symptoms but also from latently infected horses[2, 6, 9, 29]. Before mating, we checked the vaginal area of all mares, but symptoms of ECE were not found. The vaginal area was washed as in previous studies [2, 6]. In those previous studies, washing the vaginal area reduced the risk of transmission. In these cases, the vaginal area was washed with hypochlorous acid solution. Virucidal activity of hypochlorous acid solution against EHV-3 was confirmed in my laboratory. In these cases, it was suspected that EHV-3 was inside the vagina and that hypochlorous acid solution did not reach the area of infection.

In 2015, two Thoroughbred stallions developed ECE symptoms and EHV-3 was isolated by penile swabs. This is the first report of EHV-3 isolation from stallions in Japan and administration of valacyclovir orally for treatment of ECE. Oral treatment with valacyclovir did not greatly shorten the duration of disease. The period for cure of the affected area depended on how long it took for papules and ulcers to heal. The affected area did not expand after administration of valacyclovir, and EHV-3 was susceptible to valacyclovir *in vitro*. Valacyclovir therefore seems to be effective for suppression of EHV-3 replication, but further investigation of the appropriate dosage of valacyclovir for treatment of ECE is required. At present, careful observation and washing of the vaginal area before mating and reducing the stress of stallions are important for prevention of ECE.

Table 2.1.1 Serum EHV-3 neutralizing antibody titers in EHV-3-infected stallions.

Stallion	Serum Collection Dates in 2015					
	Apr. 11	Apr. 27	May 27	Jun. 30	Jul. 27	Aug. 31
A	<2	4	2	8	8	8
	May 15	Jun. 3	Sep.16			
B	<2	2	16			

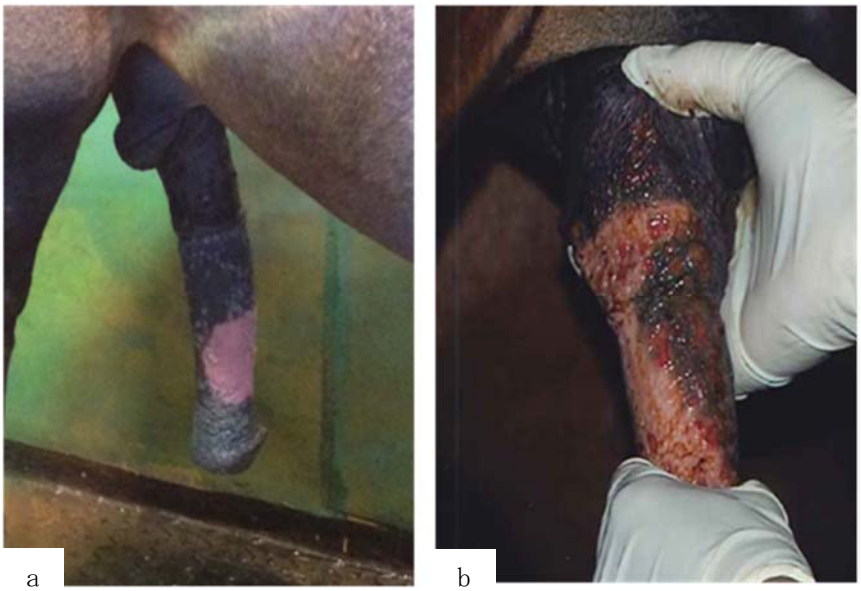

Figure 2.1.1 Symptoms of penile area.

(a): Stallion A on the 5th day after onset. (b): Stallion B on the 3rd day after onset.

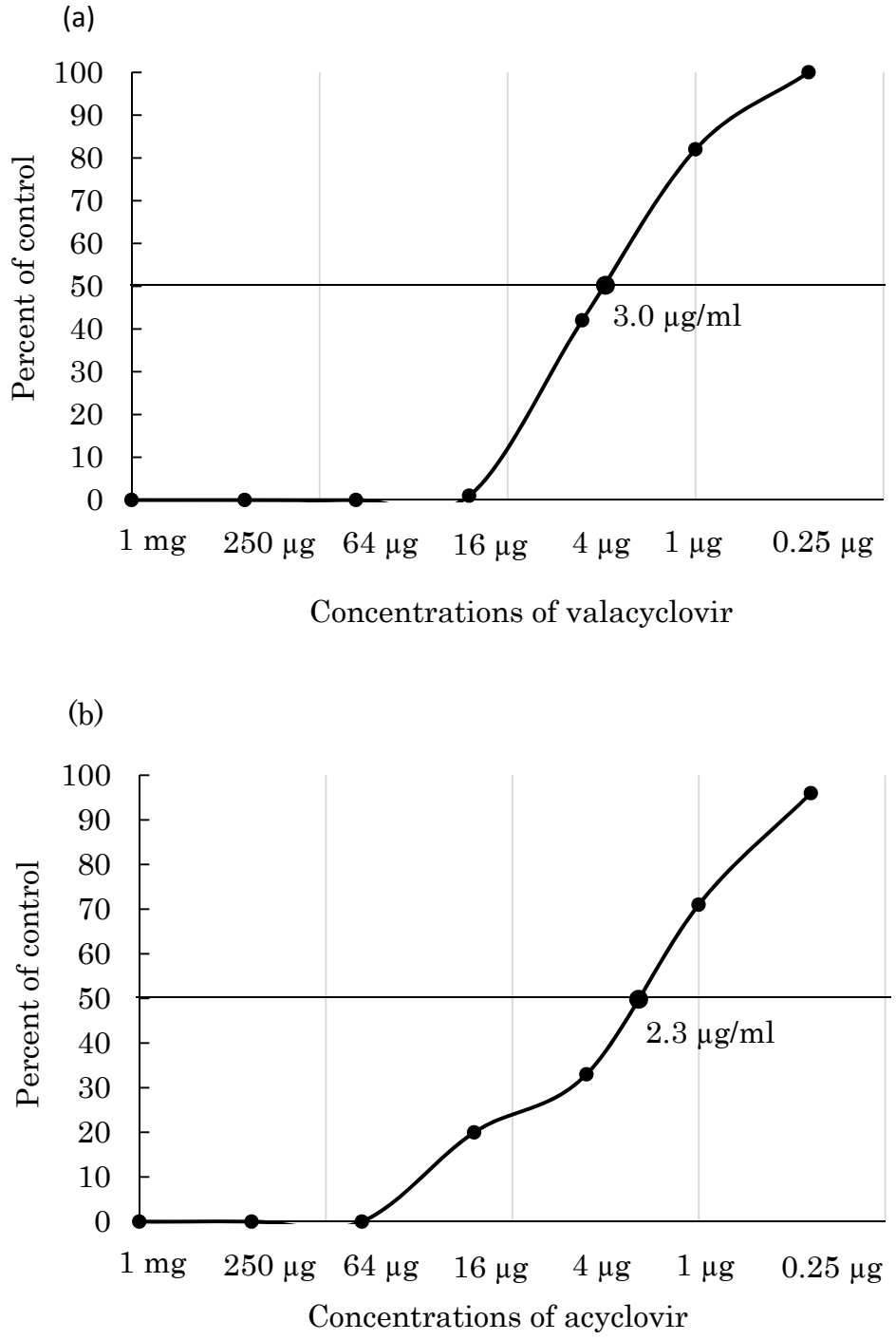

Figure 2.1.2. Dose-response curves of valacyclovir (a) and acyclovir (b) for equine herpesvirus 3. EC_{50} values are 3.0 µg/ml and 2.3 µg/ml for valacyclovir and acyclovir, respectively.

Section 2. Isolation of equine herpesvirus 3 (EHV-3) from equine coital exanthema

of two stallions and sero-epidemiology of EHV-3 infection in Japan

2.2.1. Introduction

ECE caused by EHV-3 is an acute, infectious, predominantly venereally transmitted disease as described in section 1. EHV-3 does not cause viremia and is non-abortigenic under natural conditions. EHV-3 showed no serologic cross-reactivity with other equine herpesviruses in neutralization tests [6, 36, 89]. Recently, the complete EHV-3 genomic sequence has been reported. It has a total size of 151,601 nucleotides and encodes 76 distinct genes [84]. EHV-3 is highly host-specific and has been grown only in cells of equine origin [16]. EHV-3 is restricted to the epithelium of the nasal and vaginal mucosa, and the virus neither breaches the basement membrane nor infects individual immune cells [69]. EHV-3 might establish a latent infection in an undefined site, from where reactivation and shedding might occur [2, 8, 9, 17, 90]. New outbreaks of disease might occur by reactivation of latent virus in clinically normal infected carrier animals [9, 90].

EHV-3 was first isolated in 1968 concurrently in the U.S.A., Canada, and Australia [15, 35, 73] and has been shown to be distributed worldwide [6]. In Japan, although symptoms similar to those ECE have occasionally been observed, the disease is obviously underdiagnosed, either because it is mild or because virological examinations at appropriate times are not performed. There has been only one report concerning isolation of EHV-3 from affected horses in Japan [81]. In this report, isolation of EHV-3 from two stallions described in section 1 and molecular characterization of the isolated viruses are described. Furthermore, by using one

of the isolated viruses, a sero-epidemiological survey was conducted in horses in Hokkaido, Japan.

2.2.2. Materials and Methods

Cell culture and virus

For virus isolation and SN tests, FHK cells were cultured as described in section 1.

Samples from affected horses

For virus isolation and DNA extraction, swabs were obtained from penile lesions in Stallion A (case 1) at Farm A and stallion B (case 2) at Farm B as described in section 1.

Virus isolation

Viruses were isolated as described in section 1.

DNA extraction

DNA was extracted from virus-infected cells as previously described [38]. Viral DNA was extracted from the supernatant fluids of swab samples described above and from isolated viruses in cell-cultured fluids using a DNeasy Blood and Tissue kit (Qiagen K.K., Tokyo, Japan).

Restriction enzyme digestion

The DNAs were digested to completion with restriction endonuclease *Bam*HI under conditions recommended by the manufacturer (Takara Bio Inc., Tokyo). The digested fragments were electrophoresed through 0.7% agarose at 30 V for 15 hr. The gels were stained with ethidium bromide (0.5 μg/ml) and visualized by transillumination at 302 nm [45].

Polymerase chain reaction (PCR) for detection of EHV-3

To detect EHV-3 DNA in swab samples, isolated viruses and the reference virus, PCR assays targeting the gG gene were carried out using Expand High Fidelity PCR System (Roche Diagnostics GmbH, Mannheim, Germany) and primers listed in Table 2.2.1 as described by Dynon et al. [27]. The PCR products were purified and sequenced as described below.

Sequencing

Regions coding for gB, gC, and VP13/14 and partial terminal regions of *Bam*HI-A and adjacent fragments were amplified by PCR with KOD-Plus-Neo (Toyobo Co., LTD., Osaka, Japan) and each of the specific primers listed in Table 2.2.1. The PCR products were purified with a High Pure PCR Product Purification kit (Roche Diagnostics GmbH) or with a QIAquick Gel Extraction kit (Qiagen K.K.) when extra bands were observed. The purified PCR products were used for sequencing. Sequencing was conducted in Hokkaido System Science Co., Ltd. (Sapporo, Japan) using the specific primers and walking primers. Sequence analyses were conducted by DNASIS Pro (Hitachi Software Engineering Co., Ltd,

Tokyo, Japan). DDBJ accession numbers assigned to the gene sequences of the analyzed isolates and Iwate-1 strain are LC189056-LC189067, LC189551-LC189554 and LC199967-LC199968.

Serum samples

For a sero-epidemiological survey, sera from eight farms (A to H) in Hokkaido were used. Farms A and B were stud farms and the others were breeding farms. Sera were collected from 44 stallions in Farm A and 17 stallions in Farm B on April 27, 2015 and June 3, 2015, respectively. Additionally, sera were collected from117 mares in Farm C, 39 mares in Farm D and 30 mares in Farm E on April 29, 2015, February 10, 2014 and January 13, 2015, respectively. Sera were also collected from 30 mares in Farm F, 25 mares in Farm G and 17 mares in Farm H on March, in 2014, January 26, 2015 and October 26, 2011, respectively. When a horse in Farm A or C was considered positive by serology, we used elapsed sera that had been stocked in my laboratory since 2002 to estimate the EHV-3 infection time.

SN test

Antibodies against EHV-3 in sera were determined by the SN test using the SS-1 isolate from stallion A as described in section 1.

2.2.3. Results

Virus isolation and identification

ECE was strongly suspected because of the clinical symptoms observed in the two affected stallions, A and B, in Farm A and Farm B, respectively [101]. Cytopathic effects (CPE) were observed in FHK cells after one-day post inoculation of penis swab samples from both stallions. CPE of both isolates were characterized by rounded cells and syncytial formation. The isolates from stallions A and B were designated as SS-1 and YS-1 strains, respectively. By PCR amplification of the partial gG gene of EHV-3, identical products with the same estimated size, approximately 520 bp, were detected from DNAs of penis swab samples of both stallions and isolated viruses. The nucleotide sequence identities of the amplified products of SS-1 and YS-1 were 99.18% and 99.80%, respectively, against that of the Japanese reference strain Iwate-1 and 100% and 99.39%, respectively, against that of full-genome sequenced Argentina isolate AR/2007/C3A (Fig. 2.2.1). *Bam*HI restriction profiles revealed that SS-1 and Iwate-1 showed almost the same pattern (Fig. 2.2.2). A remarkable difference between SS-1 and YS-1 profiles was observed in the *Bam*HI-A fragments. The *Bam*HI-A fragment of YS-1 was larger than those of SS-1 and Iwate-1 by approximately 1.8 kbp. To clarify the size difference in *Bam*HI-A fragments between SS-1 and YS-1, Sequence analysis was conducted on partial terminal regions of *Bam*HI-A fragments. Based on the *Bam*HI restriction map [92], the *Bam*HI-A fragment consists of a unique short (Us) segment bracketed by a part of the inverted repeat sequences (Fig. 2.2.3). PCR primers were designed within the repeat sequences to amplify the estimated size of 1.2 kbp of both terminals of the *Bam*HI-A fragment of YS-1. These primers could also amplify the same size of PCR products in SS-1. Sequence analysis of both PCR products

revealed that one *Bam*HI restriction site, which was located at 958 bp from the 5' end of the SS-1 PCR products, was missing in the YS-1 PCR product (Fig. 2.2.3). Since this site was within both inverted repeat sequences, the size difference between SS-1 and YS-1 *Bam*HI-A fragments was 1,916 bp. From these results, SS-1 and YS-1 were identified as EHV-3, but *Bam*HI profiles of the two viruses were slightly different.

Sequence analysis

SS-1 and YS-1 were isolated at almost the same time in the same prefecture. In order to determine whether SS-1 and YS-1 were similar or not, complete nucleotide sequences of gB, gC and VP13/14 were determined and compared, and the sequences were also compared to those of Iwate-1 and Argentina isolate AR/2007/C3A. The gB nucleotide sequences and deduced amino acid sequences of SS-1, YS-1 and Argentina isolate were the same, 100% identities, but not identical to those of Iwate-1, 99.77% and 99.80% identities, respectively (Table 2.2.2). The gC nucleotide sequence and deduced amino acid sequence of SS-1 were not identical to those of the other three viruses, 99.79% to 99.93% identities and 99.58% to 99.79% identities, respectively (Table 2.2.3). VP13/14 nucleotide sequences and deduced amino acid sequences of SS-1 and YS-1 were not identical, 99.89% and 99.77% identities, respectively (Table 2.2.4). The results showed that SS-1 and YS-1 were similar isolates and also similar to Iwate-1 and Argentina isolate.

Sero-epidemiology

To examine the prevalence of EHV-3 antibody in Japanese horses, SN tests using SS-1 strain and a total of 319 sera from the two stallion farms (Farms A and B) and six mare farms (Farms C to H) were conducted (Table 2.2.5). In Farms A and B, positive rates were 9.1% and 17.6%, respectively. Three of the six mare farms showed positive rates ranging from 2.6% to 3.4%. The positive rate for stallions (11.5 %) was higher than that for mares (2.3%). To estimate the EHV-3 infection time of EHV-3 antibody-positive horses, a retrospective study was conducted in three stallions in Farm A and one mare in Farm C (Table 2.2.6). My laboratory has been stocking sera from horses in Farms A and C since 2002. Stallion No. 411 showed sero-conversion in June 2011 and May 2014. Stallion No. 825 showed sero-conversion in August 2013. Stallion No. 833 showed sero-conversion in May 2013. Mare No. 263 showed sero-conversion in May 2012. These four horses showed sero-conversion in the breeding season.

2.2.4. Discussion

In this study, two EHV-3 strains were isolated from separate ECE cases of two stallions in Japan. The *Bam*HI DNA profile of YS-1 was almost the same of that of the prototype of EHV-3 [92]. Similar *Bam*HI DNA profiles of SS-1 and Iwate-1 were observed in field EHV-3 isolates in Australia and the U.S.A. [13, 43]. The remarkable size difference observed between *Bam*HI-A fragments of YS-1 and SS-1 was due to the lack of two *Bam*HI restriction sites in YS-1, each positioned at 958 bp from both ends of the *Bam*HI-A fragment of YS-1. Sequence analyses of gG,

gB, gC and VP13/14 revealed that SS-1, YS-1 and Iwate-1 were slightly different. These results suggested that there might be several types of EHV-3 circulating in the horse population in Japan.

Barrandeguy reported that there were at least four genetically distinguishable EHV-3 strains based on the partial sequence of the gG gene obtained from 25 field isolates [5]. Three base substitutions in the gG gene have been found at positions 904, 1103 and 1264, which result in strains CAT (Australia), AAT (U.S.A. and Brazil), CAG (Argentina) and ACT (Argentina). SS-1 belongs to the CAG type, and YS-1 and Iwate-1 belong to the ACT type. Therefore, in these criteria, Japanese EHV-3 isolates belong to the same group that Argentina isolates belong to, but are different from isolates of the U.S.A., Brazil and Australia.

The sources of EHV-3 infection in the two stallions were unclear. In both stallion farms, the female progenital skin and mucosa were carefully observed before mating [101]. One possible reason was that the horse initiating the infection might not have shown clinical signs, and careful examination might have revealed one or few ulcerative lesions inside the progenital mucosa from which reactivated virus was shed and transmitted to the mating partner [90]. Epidemiological data suggested that the original viral source of an outbreak of ECE might be either a visiting mare brought onto the stud farm for breeding or virus reactivated from a member of the resident stallion or mare population [11, 17, 34, 81].

The sero-epidemiological study revealed that the EHV-3 positive rate was 4.1% and that EHV-3 infection might be widespread in the horse population in Japan. In Argentina, almost 50% of adult, breeding-age mares were seropositive

and presumably latently infected [9]. In Mongolia, EHV-3 antibody-positive horses were detected in most provinces and the positive rate (22.9%) was higher than that in this study [71]. One of the reasons for this high rate might be that horse reproduction in Mongolia occurred through natural mating and the rate of artificial insemination was low.

This study showed that the infection rate of stallions was higher than that of mares. This suggested that infection risk for stallions might be higher than that for mares due to mating with many mares. In the retrospective study, all of the four EHV-3 antibody-positive horses showed sero-conversion in the breeding season. One stallion (No. 411) showed sero-conversion against EHV-3 twice without any clinical symptoms of ECE. The antibody-positive period after the first sero-conversion was only 2 months, but that after the second sero-conversion was at least 1 year, suggesting that reinfection or reactivation of EHV-3 might have occurred. Furthermore, although two other stallions (Nos. 825 and 833) in Farm A and 1 mare (No. 263) in Farm C showed sero-conversion against EHV-3 during the rearing period in each farm, no clinical case of ECE was observed. These results suggested that asymptomatic infection might have occurred frequently. In Japan, it was reported that sero-conversion against EHV-3 was observed despite no development of progenital regions in stallions and mares [81]. Without mating with a stallion, the antibody titer against EHV-3 increased in some antibody-positive mares [81]. These increases of the antibody titer might be due to reactivation of EHV-3 like other latent herpesviruses [2, 51]. Barrandeguy et al.[7, 9] reported that latently infected mares may not only reactivate the virus and shed it but also

be reinfected and shed a second virus to other horses. Viral excretion without clinical signs can therefore occur and contribute to the venereal and mechanical transmission of EHV-3.

In conclusion, EHV-3 was firstly isolated from ECE of two stallions reared in different farms in Japan. The results of sero-epidemiological survey suggested that EHV-3 infection might exist widely without clinical symptoms in Japanese horses and that infection might occur mainly in the breeding season in Japan.

Table 2.2.1 Primers used in PCR amplification.

Target gene	Primer	Sequence	Location	PCR product size (bp)
gG	gG-F	GCGCTCTCTCGGCCTTGCCAG	132949-132969[a]	518
	gG-R	GGCGTCTCGAAAAGCGAGAG	133466-133447	
gB	gB-F	TTCTCCTCGGTTTTCCACTG	60484-60503	3,159
	gB-R	TGTCGGATACGCGTAATGTT	63642-63623	
gC	gC-F	TAATCGAGATCGGCGAGTTG	20328-20347	1,531
	gC-R	GCACGAAACCCTGTTTGC	21858-21841	
VP13/14	13-F	TGCGCTTTCGTCTGTGATAC	14285-14304	2,766
	13-R	GGGTAGAGGCGCACAAAAG	17050-17032	
*Bam*HI-A	A-F	AAGAGGAGTGTAAGCGAAAGGA	125328-125349	1,180
			143754-143733	1,180
	A-R	TAGCCCATCGCGTAGAAATC	126507-126488	
			142575-142594	

a. Location at the complete nucleotide sequence of EHV-3 strain AR/2007/C3A

(Genbank NC_024771.1).

Table 2.2.2 Identities of nucleotide sequence and amino acid sequence of gB among EHV-3 isolates.

	Identity (%) of nucleotide sequence (2,982 bp)			
	SS-1	YS-1	Iwate-1	AR/2007/C3A
SS-1[a]		100.00	99.77	100.00
YS-1[b]	100.00		99.77	100.00
Iwate-1[c]	99.80	99.80		99.77
AR/2007/C3A[d]	100.00	100.00	99.77	
	Identity (%) of amino acid sequence (994 aa)			

a. EHV-3 isolate from case 1.
b. EHV-3 isolate from case 2.
c. EHV-3 isolate from Iwate prefecture [81].
d. Argentina isolate (KM051845.1).

Table 2.2.3 Identities of nucleotide sequence and amino acid sequence of gC among

EHV-3 isolates.

	Identity (%) of nucleotide sequence (1,419 bp)			
	SS-1	YS-1	Iwate-1	AR/2007/C3A
SS-1[a]		99.93	99.79	99.79
YS-1[b]	99.79		99.86	99.86
Iwate-1[c]	99.58	99.79		99.72
AR/2007/C3A[d]	99.79	100.00	99.79	
	Identity (%) of amino acid sequence (473 aa)			

a. EHV-3 isolate from case 1.
b. EHV-3 isolate from case 2.
c. EHV-3 isolate from Iwate prefecture [81].
d. Argentina isolate (KM051845.1).

Table 2.2.4 Identities of nucleotide sequence and amino acid sequence of

VP13/VP14 among EHV-3 isolates.

	SS-1	YS-1	Iwate-1	AR/2007/C3A
		Identity (%) of nucleotide sequence (2,658 bp)		
SS-1[a]		99.89	99.81	99.92
YS-1[b]	99.77		99.85	99.81
Iwate-1[c]	99.66	99.89		99.74
AR/2007/C3A[d]	100.00	99.77	99.66	
		Identity (%) of amino acid sequence (886 aa)		

a. EHV-3 isolate from case 1.
b. EHV-3 isolate from case 2.
c. EHV-3 isolate from Iwate prefecture [81].
d. Argentina isolate (KM051845.1).

Table 2.2.5 Prevalence of EHV-3 neutralizing antibody in horses in Hokkaido in Japan.

Farm	Category	Collecting date	Positive sera/tested sera
A	Stallion	27 Apr. 2015	4[a] (4 - 16)[b]/44(9.1)[c]
B	Stallion	3 Jun. 2015	3[d] (2-16) /17(17.6)
C	Broodmare	29 Apr. 2015	4 (2 - 8) /117(3.4)
D	Broodmare	10 Feb. 2014	1 (4) /39(2.6)
E	Broodmare	13 Jan. 2015	0/30(0)
F	Broodmare	11 Mar. 2014	1(8)/30(3.3)
G	Broodmare	26 Jan. 2015	0/25(0)
H	Broodmare	26 Oct. 2011	0/17(0)
Subtotal	Stallion	-	7/61(11.5)
	Broodmare	-	6/258(2.3)
Total			13/319(4.1)

a. One positive serum is from stallion A.
b. Numbers in parenthesis are the range of EHV-3 neutralizing antibody-positive titers
c. Number in parenthesis is positive percent.
d. One positive serum is from stallion B.

Table 2.2.6. Retrospective study of EHV-3 neutralizing antibody positive-horses in Farms A and C.

Farm	Horse No.	Neutralizing antibody titer															
A	411 (♂, 23y)[c]	02[a]	'07	'08	'09	'10	'11				'12	'13	'14				'15
		7[b]	7	7	7	10	5	6	7	8	6	6	4	5	7	12	4
		-[d]	-	-	-	-	-	8	2	-	-	-	-	32	16	8	16
	825 (♂, 8y)	'10	'11	'12	'13				'14	'15							
		10	7	10	6	7	8	9	12	4							
		-	-	-	-	-	4	4	8	4							
	833 (♂, 10y)	'11	'12	'13			'14	'15									
		7	10	4	5	6	12	4									
		-	-	-	32	16	8	16									
C	263 (♀, 14y)	'07	'08	'11	'12			'13	'15								
		6	7	12	4	5	6	9	4								
		-	-	-	-	8	4	2	2								

a. Year, b. Month, c. Inside of parenthesis shows gender and age (years old), d) -: <2

Iwate-1	813	GGGCCCTCAATCCCTGCTCA TTGGCGCCCTGGGACTTCGC ATCCTGAGCCAGCGCTGGTA CATGCTGCCGGGCGAGACGT ACGACCAGCT	902
SS-1	813	....A....	902
YS-1	813	....A....	902
AR/2007/C3A	813	....A....	902
Iwate-1	903	GAGGCAAAATTCCAGAGGGT CTGCCCGCGGCGCAGACAGG GAATCCGCGGCCGACGTAAC AGAACCCGAAGAAAAGCCTT CGGAAAAAAC	992
SS-1	903	.C.....	992
YS-1	903	....	992
AR/2007/C3A	903	.C.....	992
Iwate-1	993	CCCCGCTTCTCCCACCGATG ACGAGGAGAAAGAAGAAGAG GAAAACGGGGATAACGAGCC AACCCCAGCGCCACCGGCCC CGGGATGCGA	1082
SS-1	993	....	1082
YS-1	993	....	1082
AR/2007/C3A	993	....	1082
Iwate-1	1083	CGAGCAAGACGCACCTGCCG CCGGAGACGGATCTCCGTGG TACACCGGCGGCATTCTCGT GTCCGGTCCTGAGTGCGGAC AGCAGAAGGG	1172
SS-1	1083	..... A.....	1172
YS-1	1083	....	1172
AR/2007/C3A	1083	..... A.....	1172
Iwate-1	1173	GACCAACTACGCGGGTATCG GCTTTCTCATCTTGGGAGTG TGTCTCCTCATCGGCCTCAT TGTCTACGTTTGCGTCCTGC GGTCCAGAGT	1262
SS-1	1173	....	1262
YS-1	1173	....	1262
AR/2007/C3A	1173	....	1262
Iwate-1	1263	CTCCGAGCGCAAGCTCCACA ACAGCTACTCTCGCTTTT	1300
SS-1	1262	.G.....	1300
YS-1	1262	....	1300
AR/2007/C3A	1263	.G.....	1300

Figure 2.2.1 Comparison of nucleotide sequences of the partial gG gene from two field strains (SS-1 and YS-1) with those of Japanese reference strain Iwate-1 and Argentina AR/2007/C3A strains.

The identical nucleotide is indicated by a dot. Numbers on the left and right sides are the nucleotide position of the EHV-3 gG gene.

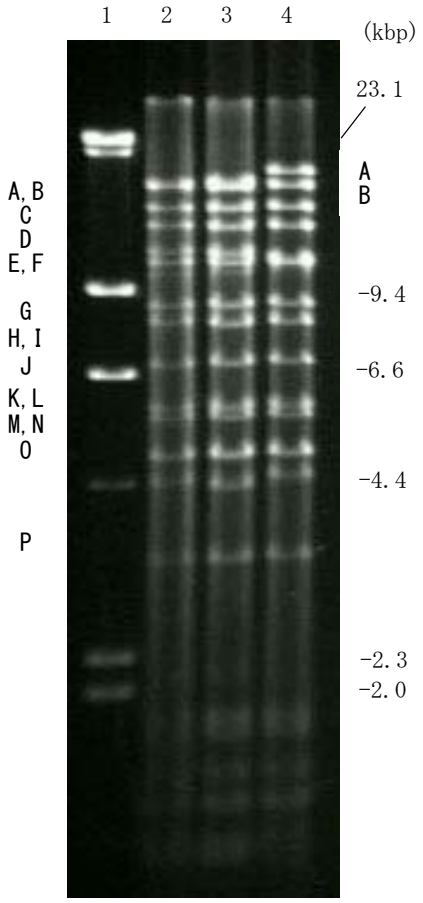

Figure 2.2.2 *Bam*HI restriction profiles of DNAs of SS-1 and YS-1 strains and Japanese reference strain Iwate-1.

Lane 1: molecular weight marker, lambda DNA *Hin*d III digest, Lane 2: Iwate-1, Lane 3: SS-1, Lane 4: YS-1.

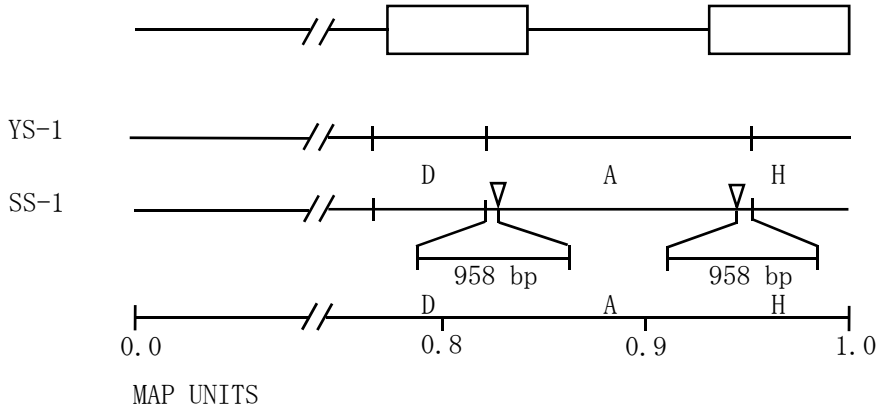

Fig. 2.2.3 *Bam*HI partial restriction map of SS-1 and YS-1 strains.

The map was based on Sullivans *et al.* [92]. Internal repeat (IR) and terminal repeat (TR) are indicated by open boxes. Extra *Bam*HI sites observed in SS-1 are indicated by open triangles.

Summary

In this dissertation, equine herpesvirus 3 (EHV-3) was isolated from 2 stallions. It was the first report of isolation of EHV-3 from stallions in Japan.

In order to evaluate the efficiency of valacyclovir, an anti-herpesvirus agent, 2 stallions were administered valacyclovir orally. It was suggested that valacyclovir was effective to suppress EHV-3, however, it did not shorten the time until the symptoms were cure. The dosage regimen used in this study seemed to result in a plasma concentration that did not exceed the 50% effective concentration (EC_{50}) value of EHV-3.

EHV-3 isolates from 2 stallions and Iwate prefecture were not the same type by sequence analysis.

In sero-epidemiology study, EHV-3 positive rate was 4.1% in Japan. Positive rate in stallions (11.5%) was higher than that of mares (2.3%). Sero-conversion against EHV-3 was observed in breeding season without any clinical symptoms of equine coital exanthema (ECE). Reinfection or reactivation of EHV-3 might occur in breeding season.

CONCLUSION

First, the present study confirmed that chemiluminescent enzyme

immunoassay (CLEIA) system is an effective tool for measurement of progesterone (P), estradiol (E) and testosterone (T) concentrations in clinical cases in horses. Further, the CLEIA system has an advantage that it allows the use of whole blood. Rapid measurement of P concentrations by CLEIA system is helpful to diagnose the estrus condition of mares coming to stud for mating. Measurement of E concentrations by CLEIA system is expected to be a help for rapid diagnosis of placental abnormalities. Measurement of T concentration by CLEIA system is helpful to diagnose reproductive abnormalities by human chorionic gonadotrophin (hCG) challenge test or gonadotropin releasing hormone (GnRH) challenge test. The rapidity of CLEIA system in T measurement is also valuable especially for monitoring T concentrations in stallions treated with T product for low libido.

Second, the present study described two cases of equine coital exanthema (ECE) and a sero-epidemiological study. An anti-herpesvirus agent, valacyclovir, was found to suppress viral multiplication in vitro, however, treatment with valacyclovir failed to improve clinical outcomes, probably due to improper timing of administration or insufficient dosage. Sero-epidemiological study revealed that equine herpesvirus 3 (EHV-3) infection exists without clinical signs in Japan. The antibody prevalence rate against EHV-3 was higher in stallions than mares and it is suggested that infection risk for stallions is higher because stallions may have an increased chance of mating with affected mares. Careful observation of external genitalis and penile area is needed in studs to prevent spreading this disease.

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