FULL PAPER *Bacteriology*

Evaluation of the Field Application of PCR in the Eradication of Contagious Equine Metritis from Japan

Toru ANZAI[1)], Ruichi WADA[1)], Toshio OKUDA[2)] and Toshihisa AOKI[2)]

[1)]Epizootic Research Station, Equine Research Institute, Japan Racing Association, 1400–4 Shiba, Kokubunji-machi, Shimotsuga-gun, Tochigi 329–0412 and [2)]Hidaka Livestock Hygiene Service Center, Kousei-cho, Shizunai-cho, Shizunai-gun, Hokkaido 056–0005, Japan

(Received 27 February 2002/Accepted 30 July 2002)

ABSTRACT. The effectiveness of the polymerase chain reaction (PCR) as a field application test for the eradication of contagious equine metritis (CEM) was evaluated. Seven-thousands five-hundred and thirty-four genital swabs were collected from 4,026 Thoroughbred broodmares and stallions in Japan to test "high risk" horses as well as for general surveillance testing from 1998 to 2001. Bacterial isolation as well as PCR testing of original specimens and cultured specimens was performed for detection of *Taylorella equigenitalis* from genital swabs. As a result, *T. equigenitalis* was detected in 12 mares and 1 stallion by PCR, although the bacteria were isolated from only 2 of the PCR-positive mares. CEM-infected and carrier horses were treated by a combination of chemotherapy and surgery. Subsequent follow-up testing over a 3-year period did not detect *T. equigenitalis*. It was demonstrated that PCR testing was more sensitive than isolation as a method for the detection of *T. equigenitalis* from genital swabs of horses in the field. It was therefore suggested that a combination of PCR testing and treatment were useful measures in the eradication of CEM from Japan.

KEY WORDS: CEM, equine, PCR, *Taylorella equigenitalis*, Thoroughbred.

J. Vet. Med. Sci. 64(11): 999–1002, 2002

Contagious equine metritis (CEM) is a highly contagious, bacterial venereal disease of horses caused by *Taylorella equigenitalis*. The disease can cause short-term infertility and severely affect the breeding operation of horses. Since the first reported outbreaks in England [5] and Ireland [14] in 1977, CEM has been recorded in many European countries, North and South America, Australia and Japan [4, 6, 10, 12]. An outbreak of CEM in the Hidaka districts, Hokkaido was first described in 1980 [6], although serological evidence suggests that CEM had infected the horse populations of Japan prior to 1978 [7]. Since then, although CEM has been eradicated from Thoroughbred populations in the United States, Australia and almost European countries, the disease continues to be a problem among Thoroughbred breeders in Japan, causing considerable economic loss. Despite bacterial isolation testing of all broodmares and stallions in main Thoroughbred breeding areas of Japan every inter-breeding season since 1980, occurrence of CEM has persisted and *T. equigenitalis* has been isolated every year to date [8, 9].

T. equigenitalis, a gram negative, nonmotile, coccoid rod, colonizes the surface of the endometrium of the uterus [15], causing endometritis and sterility in mares. The organism may then persist on the clitoris to occasionally infect the penis of stallions that cover the mare [13]. Diagnosis of CEM is therefore performed by bacterial isolation from the cervix of the uterus, the clitoral sinus, and the clitoral fossa of mares as well as the urethral orifice, the urethral fossa and the penile sheath of stallions [16]. Neverthless, because of the microaerophilic, drug sensitive and slow-growing properties of *T. equigenitalis*, visible colony formation usually takes 4 to 6 days, and is often obscured by the growth of other quick-growing bacteria contaminating the sample [11].

The purpose of this paper was to apply PCR testing to the diagnosis of CEM in the field, and evaluate its efficacy in the eradication of this disease from Japan. We have developed a PCR test based on species specific DNA sequences of *T. equigenitalis*, and demonstrated its rapidity, specificity and high sensitivity to be useful in the detection of *T. equigenitalis* from genital swabs of horses [3]. PCR testing, therefore, overcomes the problems of isolation testing as a diagnostic method for CEM and may increase the rate of detection of infected or carrier horses in the field.

MATERIALS AND METHODS

Genital swabs: Swabs of the cervix of the uterus, the clitoral fossa and the clitoral sinuses of mares, as well as the urethral orifice, the urethral fossa and the penile sheath of stallions were taken with commercial swabbing and transportation kits. MINI-TIP CULTURETTE™ (Becton Dickinson Microbiology Systems, MD, U.S.A.), CultureSwabPlus™ Mini-Tip (Becton Dickinson Microbiology Systems) and TRANSWAB E.N.T. (Medical Wire & Equipment Co., Ltd., Wilts. U.K.) were used for swabbing the clitoral sinus, whereas CULTURETTE™ (Becton Dickinson Microbiology Systems) and TRANSWAB (Medical Wire & Equipment Co., Ltd.) were used for other genital sites. Genital swabs were transported to the Livestock Hygiene Service Center for bacterial isolation and to the Epizootic Research Station, Equine Research Institute, Japan Racing Association for PCR testing.

Swabbing scheme: "High risk" horses were defined as those horses from which *T. equigenitalis* had been isolated within the last 3 years. Genital swabs were taken annually from "high risk" horses 3 times prior to every breeding season, until negative results were demonstrated for 3 succes-

sive years. For surveillance, genital swabs were collected from "suspected" horses which were defined as horses suspected with genital infection, had a low conception rate or had mated with a carrier horse during the breeding season in Hidaka or Iburi district in Hokkaido Prefecture or Kagoshima Prefecture.

Bacterial isolation: Genital swabs were inoculated onto Eugon chocolate agar (ECA) supplemented with 200 μg /m*l* streptomycin (ECAS+) [11]. Cultures were incubated at 37°C in an atmosphere containing 10% CO_2 for 6 days. Identification of isolates was done as described previously [6].

PCR testing: The PCR protocol used in this study was a modified method of that described previously [2,3]. Genital swabs were each suspended in 500 μl of PBS (pH 7.2) and centrifuged at 12,000 rpm for 1 min. Almost all of the supernatant was then discarded and the pellets were resuspended in a small volume of residual supernatant (about 20 μl). Approximately half of the sample volumes were inoculated onto ECAS+ and incubated at 37°C in a microaerophilic atmosphere containing 10% CO_2 for 4 to 6 days. The remaining half of each sample was added to 100 μl of InstaGene™ Matrix (BIO-RAD Laboratories, CA, U.S.A.) for bacterial DNA isolation. Bacterial DNA was then extracted according to the manufacturer's instructions. For direct 2-step semi-nested PCR (direct PCR), initial PCR was performed in a 50 μl volume containing 5 μl of bacterial DNA solution, 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 0.4 μM (each) of P1 and N2 PCR primer [3], and 1.25 U of Z-Taq™ (TAKARA SHUZO Co., Ltd., Shiga). Samples were amplified by one denaturation step at 95°C for 3 min followed by 30 amplification cycles consisting of denaturation at 98°C for 1 sec, annealing at 66°C for 5 sec, and elongation at 72°C for 3 min in TaKaRa PCR Thermal Cycler MD (TAKARA SHUZO Co., Ltd.). The resultant PCR product was amplified in a secondary semi-nested PCR with a second PCR primer set, P2-N2, under the same reaction conditions as described. Amplicons were analyzed by 2% agarose gel electrophoresis. PCR testing of cultured samples (culture PCR test) was performed on swabs of samples cultured on ECAS+ (described above). Cultured samples underwent DNA extraction and PCR amplification as described above for direct PCR.

Treatment: Isolation and/or PCR (direct and/or culture) positive horses were treated with methods described previously [1]. Briefly, the clitoris of carrier mares was washed with disinfectant (gluconate chlorhexidine) and the uterus was treated with antibiotics (gentamicin) daily for 5 or more days. Finally, the clitoral sinus was surgically removed. Local chemotherapy of the uterus was performed at the same time. To confirm elimination of the organism, isolation and PCR testing were done 7 or more days after the final treatment. Testing was done 3 times at intervals of about 7 days. Treatment of stallions consisted of daily washing of the urethral fossa and the penile sheath with disinfectant for 3 or more days. To confirm the organism had been eliminated from the penis, isolation and PCR testing were done 7 or more days after the final treatment. Testing was conducted 3 times at intervals of 2 or more days.

RESULTS

Testing of "high risk" horses: In 1998, bacterial isolation and PCR testing were performed on 27 mares at Hidaka district, 2 mares at Iburi district and 1 mare at Aomori Prefecture. As a result, 10 mares were detected as carrier mares of CEM by direct and/or culture PCR testing, although only 2 infected mares could be detected by isolation testing (Table 1). All carrier mares were treated, and elimination of *T. equigenitalis* was confirmed by PCR. In 1999, 22 "high risk" horses in Hidaka, Iburi and Aomori were examined as in 1998, with only negative results obtained. Likewise, tests were carried out on 11 horses in the year 2000 and on 8 horses in 2001, again with only negative results.

Surveillance: One-thousand-eight-hundred and seventy-two swabs taken from 1,033 "suspected" Thoroughbred broodmares and 20 "suspected" stallions at Hidaka in 1998 were examined by bacterial isolation as well as direct and culture PCR. As a result, CEM was detected by PCR in one

Table 1. Detection of *Taylorella equigenitalis* from genital swabs taken from "high risk" Thoroughbred horses

Year	Test	Genital swabs							Thoroughbred		
		Cervix of the uterus	Clitoral fossa	Clitoral sinus	Urethral orifice	Urethral fossa	Penile sheath	Total	Broodmares	Stallions	Total
1998	Isolation	0/73[a)]	0/151	2/107	0/0	0/0	0/0	2/331	2/30	0/0	2/30
	Direct PCR	1/73	6/151	5/107	0/0	0/0	0/0	12/331	7/30	0/0	7/30
	Culture PCR	1/73	6/151	5/107	0/0	0/0	0/0	12/331	7/30	0/0	7/30
	Synthesis results[b)]	2/73	8/151	7/107	0/0	0/0	0/0	17/331	10/30	0/0	10/30
1999	Synthesis results	0/45	0/72	0/39	0/3	0/3	0/3	0/165	0/21	0/1	0/22
2000	Synthesis results	0/27	0/32	0/6	0/3	0/3	0/3	0/74	0/10	0/1	0/11
2001	Synthesis results	0/14	0/27	0/0	0/3	0/3	0/3	0/50	0/7	0/1	0/8
Total		2/159	8/282	7/152	0/9	0/9	0/9	17/620	10/68	0/3	10/71

a) Denominators represent the number of samples or horses tested, while the numerator indicates the number of positive samples or horses.
b) Synthesis results; represent the total number of samples which were positive by isolation, direct PCR or culture PCR testing

Table 2. Detection of *Taylorella equigenitalis* from genital swabs taken from "suspected" Thoroughbreds that were suspected of having genital infection, low conception rate or had mated with a carrier horse during the breeding season

Year	Test	Genital swab							Thoroughbred		
		Cervix of uterus	Clitoral fossa	Clitoral sinus	Urethral orifice	Urethral fossa	Penile sheath	Total	Broodmares	Stallions	Total
1998	Isolation	0/1,111[a)]	0/347	0/342	0/24	0/25	0/23	0/1,872	0/1,033	0/20	0/1,053
	Direct PCR	1/1,111	0/347	0/342	0/24	1/25	0/23	2/1,872	1/1,033	1/20	2/1,053
	Culture PCR	0/1,111	0/347	0/342	0/24	0/25	0/23	0/1,872	0/1,033	0/20	0/1,053
	Synthesis results[b)]	1/1,111	0/347	0/342	0/24	1/25	0/23	2/1,872	1/1,033	1/20	2/1,053
1999	Synthesis results	0/801	0/94	0/84	0/2	0/5	0/5	0/991	0/761	0/5	0/766
2000	Isolation	0/790	0/54	0/891	0/6	0/9	0/6	0/1,756	0/900	0/9	0/909
	Direct PCR	0/790	0/54	1/891	0/6	0/9	0/6	1/1,756	1/900	0/9	1/909
	Culture PCR	0/790	0/54	1/891	0/6	0/9	0/6	1/1,756	1/900	0/9	1/909
	Synthesis results	0/790	0/54	1/891	0/6	0/9	0/6	1/1,756	1/900	0/9	1/909
2001	Synthesis results	0/889	0/421	0/952	0/11	0/11	0/11	0/2,295	0/1,216	0/11	0/1,227
Total		1/3,591	0/916	1/2,269	0/43	1/50	0/45	3/6,914	2/3,910	1/45	3/3,955

a) Denominators represent the number of samples or horses tested, and the numerators indicate the number of positive samples or horses.
b) Synthesis results represent the total number samples which were positive by isolation, direct PCR or culture PCR testing.

Table 3. Comparison of PCR tests for detection of *Taylorella equigenitalis* from genital swabs

Detectional method	No. of positive samples[a)] (%[b)])	No. of positive horses[a)] (%[b)])
Direct PCR	15 (75)	10 (77)
Culture PCR	13 (65)	8 (62)
Synthesis results	20 (100)	13 (100)

a) Results for 4 years were totalled.
b) Percentages of synthesis results.

broodmare and one stallion (Table 2). These horses were treated and elimination of *T. equigenitalis* was confirmed by PCR. In 1999, PCR and isolation testing of 991 swabs taken from 761 "suspected" broodmares and 5 "suspected" stallions in the Hidaka district were consistently negative. In 2000, a PCR positive swab that was taken from the clitoral sinus of a "suspected" broodmare in the Hidaka district was demonstrated among 1,756 samples taken from 900 "suspected" broodmares and 9 "suspected" stallions in the Hidaka and Iburi districts of Hokkaido and Kagoshima Prefecture. This CEM carrier mare was treated and retired as a broodmare. PCR or isolation results of 2,295 swabs taken from 1,216 "suspected" broodmares and 11 "suspected" stallions in Hidaka and Iburi district in Hokkaido and Kagoshima Prefecture in 2001 were all negative.

Sensitivity of direct and culture PCR test: Comparison of each PCR test for detection of *T. equigenitalis* from genital swabs is shown in Table 3. Direct PCR detected 15 (75%) positive samples among 20 genital swabs of "high risk" and "suspected" Thoroughbred horses and 10 (77%) among 13 positive Troroughbred horses, whereas culture PCR detected 13 in 20 (65%) and 8 in 13 (62%), respectively.

DISCUSSION

Despite implementation of a number of control measures since its detection in 1980, CEM has not yet been eradicated from Japan. Three-hundred and twenty-one, 56, 34, 25, 33, 119, 98, 95, 94, 69, 23, 32, 19, 23, 11, 0, 22 and 5 Thoroughbred horses were detected with CEM each year from 1980 to 1997 (data unpublished). Recently we reviewed the methods of control of CEM that were employed in the past. It was revealed that neither diagnosis by complete swabbing of the clitoral sinus nor treatment by surgical removal of the clitoral sinus was practiced. These are thought to be factors contributing to why CEM continues to persist in Japan. *T. equigenitalis* colonizes the clitoral sinus and clitoral fossa of carrier mares, and it is difficult to completely remove the organism from the sinus by any method except surgical operation. In this study we introduced swabbing, together with surgical removal of the clitoral sinus for the treatment of CEM. These measures appeared to have had a significant effect on reducing the detection rate among carrier mares since *T. equigenitalis* could not be detected from "high risk" horses after treatment (Table 1).

As was suggested in a previous report [3], the diagnostic PCR testing for CEM demonstrated a high rate of detection among CEM affected or carrier horses. As shown in Tables 1 and 2, detection of *T. equigenitalis* by PCR from samples from all genital sites was apparently more sensitive than bacterial isolation. Among samples taken from "high risk" mares in 1998, *T. equigenitalis* was not isolated from 2 PCR positive swabs taken from the cervix of the uterus, from 8 PCR positive swabs taken from the clitoral fossa or from 5

of 7 PCR positive swabs of the clitoral sinus (Table 1). As shown in Table 2, *T. equigenitalis* was also not isolated from 1 PCR positive swab taken from the cervix of the uterus, 1 PCR positive swab taken from the urethral fossa or 1 PCR positive swab taken from the clitoral sinus. These results strongly suggest that detection of *T. equigenitalis* cannot be based solely on isolation and that PCR testing described here is a much more sensitive method for detection of *T. equigenitalis* from genital sites.

Although PCR testing was apparently much more sensitive than bacterial isolation, reduced sensitivity was observed if either direct or culture PCR was used alone (Table 3). Direct PCR was unable to detect a small number of bacterial cells (perhaps fewer than 10 cells), and culture PCR could not detect bacteria which failed to grow on ECAS+ agar. As indicated by the results, a combination of direct and culture PCR was the most sensitive method for detection of *T. equigenitalis* from genital swabs in field cases, but it is costly to use both PCR methods in routine testing for diagnosis of CEM. Because direct PCR detected 77% of cases (horses) detected by combined direct and culture PCR, and was much more sensitive than isolation, the use of direct PCR alone is a potentially useful method for field testing for CEM. We believe that CEM can be eradicated from Japan, provided all broodmares and stallions are tested by PCR during the next few years.

ACKNOWLEDGMENTS. We are grateful to Ms. J. Goma and Ms. F. Yomogida for their great technical assistance. We also thank the staff of Hidaka, Iburi, Mutu, Kagoshima-chuuoh, Aira, Soo and Kimotsuki Livestock Hygiene Service Centers for their help with isolation, and the staff of Hidaka Livestock Hygiene and Disease Self-prevention Conference and Japan Bloodhorse Breeders' Association for sampling.

REFERENCES

1. Anzai, T. 2001. Treatment of contagious equine metritis. *J. Jpn. Vet. Med. Assoc.* **54**: 18–20 (In Japanese).
2. Anzai, T., Eguchi, M., Eto, M., Okuda, T., Aoki, T., Sawada, T. and Matsuda, M. 2001. Establishment and evaluation of the PCR test for diagnosing contagious equine metritis. *J. Jpn. Vet. Med. Assoc.* **54**: 345–348 (in Japanese with English summary).
3. Anzai, T., Eguchi, M., Sekizaki, T., Kamada, M., Yamamoto, K. and Okuda, T. 1999. Development of a PCR test for rapid diagnosis of contagious equine metritis. *J. Vet. Med. Sci.* **61**: 1287–1292.
4. Bleumink-Pluym, N., ter Laak, ED A. and van der Zeijst, B. A. M. 1990. Epidemiologic study of *Taylorella equigenitalis* strains by field inversion gel electrophoresis of genomic restriction endonuclease fragments. *J. Clin. Microbiol.* **28**: 2012–2016.
5. Crowhurst, R. C. 1977. Genital infection in mares. *Vet. Rec.* **100**: 476.
6. Kamada, M., Akiyama, Y., Oda, T. and Fukuzawa, Y. 1981. Contagious equine metritis: Isolation of *Haemophilus equigenitalis* from horses with endometritis in Japan. *Jpn. J. Vet. Sci.* **43**: 565–568.
7. Kamada, M., Oda, T., Fukuzawa, Y., Ohishi, H., Wada, R., Fukunaga, Y. and Kumanomido, T. 1983. Contagious equine metritis: A survey on complement fixing antibody against *Haemophilus equigenitalis* in light horses in Japan. *Bull. Equine Res. Inst.* **20**: 119–125.
8. Matsuda, M., Miyazawa, T. and Anzai, T. 1999. Detection of a common genotype among strains of *Taylorella equigenitalis* isolated from Thoroughbred horses in Japan between 1994 and 1996. *J. Basic Microbiol.* **39**: 127–130.
9. Miyazawa, T., Matsuda, M., Isayama, Y., Samata, T., Ishida, Y., Ogawa, S., Takei, K., Honda, M. and Kamada, M. 1995. Genotyping of isolates of *Taylorella equigenitalis* from Thoroughbred brood mares in Japan. *Vet. Res. Commun.* **19**: 265–271.
10. Powell, D. G. 1981. Contagious equine metritis. *Adv. Vet. Sci. Comp.* **25**: 161–184.
11. Swaney, L. M. and Sau, S. P. 1978. CEM: Bacteriological methods. *Vet. Rec.* **102**: 43.
12. Swerczek, T. W. 1978. Contagious equine metritis in the USA. *Vet. Rec.* **102**: 512–513.
13. Timoney, P. J. 1996. Contagious equine metritis. *Comp. Immun. Microbiol. Infect. Dis.* **19**: 199–204.
14. Timoney, P. J., Ward, J. and Kelly, P. 1977. A contagious genital infection of mares. *Vet. Rec.* **101**: 103.
15. Wada, R., Kamada, M., Fukunaga, Y. and Kumanomido, T. 1983. Studies on contagious equine metritis IV. Pathology in horses experimentally infected with *Haemophilus equigenitalis*. *Bull. Equine Res. Inst.* **20**: 133–143.
16. Watson, E. D. 1997. Swabbing protocols in screening for contagious equine metritis. *Vet. Rec.* **140**: 268–271.