# Construction of a Recombinant Plasmid as Reaction Control in Routine PCR for Detection of Contagious Equine Metritis (CEM-PCR)

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ABSTRACT. Contagious equine metritis (CEM) is a highly contagious bacterial venereal disease of horses caused by *Taylorella equigenitalis*. CEM-PCR is a semi-nested PCR method for detecting this bacterium. Although this technique is regarded as a sensitive diagnostic method for CEM, there are risks of it generating false positive and false negative results. In this study, we constructed a recombinant plasmid (CEM-POS) as reaction control to assure adequate PCR reaction and prevent false positive results caused by contamination of the reaction control in routine CEM-PCR examinations. CEM-POS was constructed by insertion of *rpoB* fragments from *Rhodococcus equi* into CEM-1P, which is a recombinant plasmid that includes a *T. equigenitalis*-specific sequence region. In CEM-PCR, the size of the PCR product from CEM-POS was clearly different from the true positive PCR product. In addition, CEM-POS retained high stability under convenient storage conditions of 4°C. These results suggest CEM-POS to be a useful tool as a reaction control in routine CEM-PCR examinations.
KEY WORDS: reaction control, PCR, *Taylorella equigenitalis*.

Contagious equine metritis (CEM) is a highly contagious bacterial venereal disease of horses caused by *Taylorella equigenitalis* [11]. It can cause short-term infertility and severely affects the breeding process. Outbreaks of this disease were first confirmed in England and Ireland in 1977 [10, 12] and have since spread worldwide [8, 9]. In Japan, since an outbreak of CEM was first reported in 1980 [7], this disease has recurred every year. A single-step PCR method for detection of *T. equigenitalis* was developed by Anzai *et al.* [2]. Recently, this method was modified to a semi-nested PCR method (CEM-PCR) for dealing with a large number of routine diagnosis examinations [3]. This method targets the unique sequence motif of *T. equigenitalis* and is regarded as a far more sensitive diagnostic method for CEM than the culture method [1]. CEM-PCR has been used annually since 2001 for the detection of CEM in approximately 12,000 horses, including thoroughbred stallions and mares as a part of the eradication program of this disease in Japan.

It is thought that the PCR-based detection method is generally faster and more sensitive than culture-based methods, especially for fastidious bacteria such as *T. equigenitalis*. However, the procedure for PCR-based methods is usually more complex than that of culture-based method. In addition, there are risks of false positive results, caused by contamination of the target DNA and of false negative results, caused by inappropriate preparation for the PCR reaction and the presence of inhibitors [4, 5]. In CEM-PCR, there is a risk of false positive results being produced by contamination of the genome DNA of *T. equigenitalis* if these are used as reaction controls. To assure the adequacy of the PCR

reaction and prevent false positive results due to contamination of the reaction controls during routine CEM-PCR examination, in this study we constructed a recombinant plasmid as a reaction control.

Two conditions are required for construction of a reaction control plasmid. One is that the specific PCR product is amplified from the plasmid in the protocol of CEM-PCR, which is able to assure an adequate CEM-PCR reaction. Another is that the size of the product from the plasmid should be significantly different from the true positive PCR product; this will be able to prevent false positive results by contamination of the plasmid to samples. To satisfy these conditions, we used a recombinant plasmid (CEM-1P) from the CEM-1 strain, which is a clone of the genomic library of *T. equigenitalis* constructed by Anzai *et al.* [3]. CEM-1P, a recombinant plasmid constructed from pUC119 by insertion of a *T. equigenitalis*-specific fragment, was used to develop CEM-PCR. We also used a partial fragment of *rpoB* of *Rhodococcus equi* as an insert into CEM-1P to create a PCR product derived from the plasmid that is larger than the true positive PCR product. The recombinant plasmid derived from CEM-1P in this study as reaction control was named CEM-POS. A schematic representation of CEM-1P and CEM-POS is shown in Fig. 1.

TA cloning method was used to construct CEM-POS. Five μg of CEM-1P was completely digested by *Eco*RV (Takara Bio Inc., Shiga, Japan) for making a blunt-end site in the *T. equigenitalis*-specific region. To prepare T-vector from digested CEM-1P, addition of thymine at the blunt-end site was performed using 100 μl of mixture consisting of 1 μg of CEM-1P, 10 μl of Taq buffer (Takara Bio Inc.), 20 μl of 10 mM dTTP (Invitrogen Corp., Carlsbad, CA, U.S.A.), 15 U of rTaq polymerase (Takara Bio Inc.), plus distilled water for adjustment of volume at 70°C for 2 hr. The

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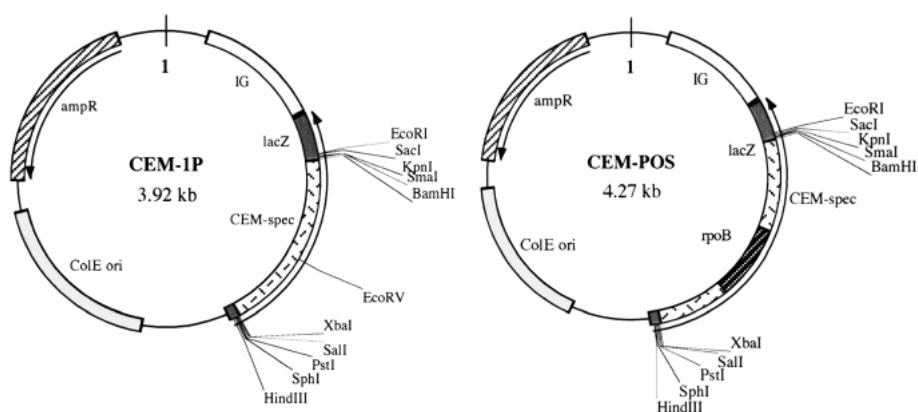    e-mail: niwa@epizoo.equinst.go.jp

Fig. 1. Schematic representation of CEM-1P and CEM-POS. CEM-spec indicates the *T. equigenitalis*-specific DNA region. The *Eco*RV cutting site of CEM-POS is deleted by insertion of a partial fragment of *rpoB* of *R. equi*.
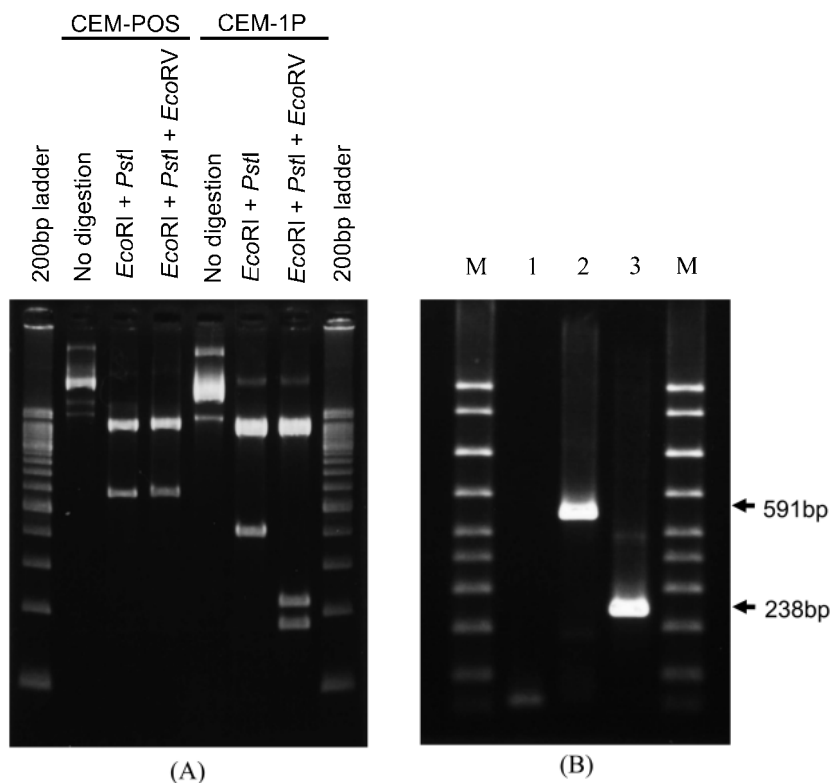


Fig. 2. Digestion by three restriction enzymes (A) and CEM-PCR (B) of CEM-POS from clone 2 and self-ligated CEM-1P. M, 100 bp ladder; 1, Negative control; Lane 2, CEM-POS; Lane 3, CEM-1P.

inserted fragment derived from *rpoB* in *R. equi* was prepared by PCR as described in a previous report [6]. Cloning and transduction was performed using a TA Cloning kit (Invitrogen Corp.) according to the accompanying instructions. Extraction of the cloning plasmid from the transformed *E. coli* was performed using a Quantum prep plasmid miniprep kit (Bio-Rad Laboratories, CA, U.S.A.)

according to the accompanying instructions. To distinguish CEM-POS from the self-ligated T-vector in TA-cloning, digestion of the extracted plasmid was performed using the restriction enzymes *Eco*RI (Takara Bio Inc.), *Eco*RV, and *Pst*I (Takara Bio Inc.). If the plasmid is a self-ligated T-vector, it is digested by all three enzymes; and if the plasmid is CEM-POS, it is digested by two enzymes, *Eco*RI and *Pst*I,
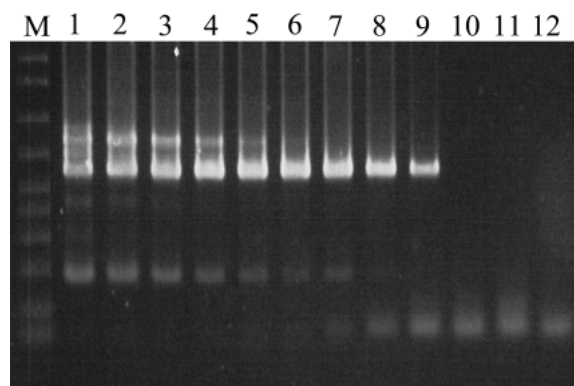
Fig. 3. Detection limit of CEM-POS. M, 100 bp ladder; Lane 1, $10^9$ copies; Lane 2, $10^8$ copies; Lane 3, $10^7$ copies; Lane 4, $10^6$ copies; Lane 5, $10^5$ copies; Lane 6, $10^4$ copies; Lane 7, $10^3$ copies; Lane 8, $10^2$ copies; Lane 9, 10 copies; Lane 10, 1 copy; Lane 11, 0.1 copy; Lane 12, Negative control. The arrow indicates an unexpected PCR product.

due to the removal of the *Eco*RV restriction site by TA cloning. The selected transformants were stored at –80°C. CEM-PCR was performed as described in a previous report [3].

After TA cloning, 34 transformed *E. coli* strains were examined. CEM-POS was introduced to 7 of the 34 of transformed *E. coli* strains, and self-ligated CEM-1P was introduced in the remainder of the transformants. Typical results of digestion of CEM-POS and self-ligated CEM-1P by the enzymes are shown in Fig. 2. As a result of digestion of CEM-POS, two prospective fragments were observed. On the other hand, three prospective fragments were observed with digestion of CEM-1P. One transformed *E. coli* harboring CEM-POS, clone-2, was selected as the candidate for the application strain. PCR product of 591 bp was produced from CEM-POS of clone-2 in CEM-PCR (Fig. 2). This product was clearly distinguishable from true positive PCR product (238 bp) via routine agarose gel electrophoresis. These data suggest that false positive results can be avoided even if samples are contaminated by CEM-POS, We therefore concluded that CEM-POS from clone-2 could be used as the reaction control.

In this study, we also evaluated the condition of CEM-POS for usage in routine CEM-PCR. We first studied the detection limit of CEM-POS by CEM-PCR using serial 10-fold dilutions of CEM-POS solution. The results of this examination are shown in Fig. 3. The detection limit of CEM-POS was around 0.05 fg (10 copies of CEM-POS).

An extra 190 bp band was observed in the PCR product from the $10^{-1}$ to $10^{-7}$ diluted solutions. This band was visibly of lower intensity than the true CEM-POS product when subjected to agarose gel electrophoresis. This band could also be distinguished from the true positive band. We thought the unexpected band had no adverse effects against usage of CEM-POS in routine CEM-PCR. We also examined the storage stability of CEM-POS at 4°C, a convenient storage temperature for routine diagnosis. The detection limit of CEM-POS ($\leq$ 100 copies) was observed to be preserved for 3 months. The detection limits of CEM-POS at 4 months later and 5 months later had decreased to 1/100 and 1/10,000, respectively. Considering these results and the safety margin needed for routine examination, we conclude that 1,000 copies of CEM-POS for each control PCR reaction and its preservation for a month at 4°C are an adequate volume and storage period for reaction controls, respectively.

In conclusion, the CEM-POS constructed in this study is a useful tool for reaction control which can assure an adequate PCR reaction and prevent false positive results caused by contamination of samples during routine CEM-PCR examination. CEM-POS has been already used for routine diagnosis examinations and quality assurance tests of CEM-PCR at Laboratory of Racing Chemistry since September 2005.

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