**Comparison of seven nucleic acid amplification tests for detection of *Taylorella equigenitalis***

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**ABSTRACT.** *Taylorella equigenitalis* causes contagious equine metritis. Here we compared seven nucleic acid amplification tests for *T. equigenitalis* to select a rapid and reliable diagnostic method. The 95% detection limits of each assay varied greatly: real-time PCR had the lowest detection limit (0.77 fg/reaction); those of some of the conventional PCRs (cPCRs) were >100 fg/reaction. In experimentally infected samples, real-time PCR and semi-nested PCR showed the highest positive numbers (33 out of 42 samples), but two of the cPCRs detected only 2 and 7 positive results. Our results indicate that the use of sensitive molecular assays is important for the efficient detection of *T. equigenitalis* in clinical samples.

**KEY WORDS:** contagious equine metritis, nucleic acid amplification test, real-time PCR, *Taylorella equigenitalis*

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*Taylorella equigenitalis* is a causative agent of contagious equine metritis (CEM), a sexually transmitted infection of horses. Infection of mares by *T. equigenitalis* is characterized by abundant mucopurulent vaginal discharge and a variable degree of vaginitis, endometritis, and cervicitis, usually resulting in temporary infertility. Stallions show no clinical signs, and asymptomatic carrier mares have been reported. CEM was first reported in England in 1977 [7, 20], and has since spread worldwide. CEM cases were first confirmed in Japan in 1980 [13] and spread to the Thoroughbred population, but was eradicated from Japanese Thoroughbred horses by 2010 as a result of comprehensive infestation control measures [1]. However, sporadic cases of CEM have been identified around the world in recent years [8, 10, 15]. The detection of *T. equigenitalis* through quarantine is most important in CEM-free countries such as Japan.

Several nucleic acid amplification tests (NAATs), including polymerase chain reaction (PCR) [3, 5, 6, 9], real-time PCR [21], semi-nested (snPCR) PCR [2], and loop-mediated isothermal amplification (LAMP) methods [14], have been developed to detect *T. equigenitalis*. NAATs are advanced diagnostic methods that are becoming more popular in place of conventional culture methods owing to their speed, efficiency, and accuracy. They have been reported to be superior to culture methods in detecting *T. equigenitalis* [4, 17]. However, their diagnostic sensitivities and usefulness for clinical specimens could vary among methods [11, 23], and it is important to use a reliable method for the detection of *T. equigenitalis* to prevent its invasion and spread into horse populations in CEM-free countries. Here, we compared the test performance of seven NAATs reportedly specific to *T. equigenitalis* DNA in three aspects: limit of detection by using high-purity DNA, clinical usefulness by using experimental clinical samples, and effectiveness for clinical samples spiked with low concentrations of *T. equigenitalis* DNA.

We selected seven NAATs: one real-time PCR, four conventional PCRs (cPCR1–cPCR4), one snPCR, and one LAMP (Table 1). The snPCR and LAMP assays were performed according to previous reports [2, 14]. The real-time PCR and cPCR assays were prepared according to previous reports [3, 5, 6, 9, 21] but using Probe qPCR Mix with UNG (Takara Bio Inc., Kyoto, Japan) for real-time PCR and using Takara Z-Taq (Takara Bio Inc.) for cPCR. Contents, conditions, and instruments of the NAATs are shown in Supplementary Tables 1, 2. Unless otherwise noted, real-time PCR was performed using StepOnePlus (Thermo Fisher Scientific, Tokyo, Japan) in combination with the BHQ1 probe. We used 29 bacterial strains to confirm detection limits and specificities, including

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five *T. equigenitalis* strains, two *T. asinigenitalis*, and other 22 strains (Table 2). Bacterial DNA was extracted with a commercial DNA extraction kit (Instagene Matrix; Bio-Rad Laboratories, Tokyo, Japan) and quantified by a Qubit 3.0 fluorometer (ThermoFisher Scientific).

All seven NAATs were designed to be specific to *T. equigenitalis*. All except for cPCR4 exclusively detected DNAs of the five *T. equigenitalis* strains derived from various backgrounds, while cPCR4 [5] detected both *T. equigenitalis* and *T. asinigenitalis* [16]. We next evaluated the detection limits of the NAATs using the five *T. equigenitalis* strains. Considering the difference in the amount of template used for each NAAT, we prepared suspension to have the same amount of DNA per reaction in any NAAT. Assays were performed three times with triplicate samples of each strain, which had been serially diluted 10-fold. The 95% detection limits of each assay (in which 95% of the samples were positive) were calculated [19]: real-time PCR had the lowest 95% detection limit (0.77 fg/reaction), followed by LAMP (0.89 fg), snPCR (6.42 fg) and cPCR1 (8.66 fg), while the limits of cPCR2 and cPCR3 were >100 fg/reaction (Fig. 1).

All NAATs except for snPCR target the ribosomal RNA (rRNA) operon (16S rRNA or 23S rRNA gene) of *T. equigenitalis*. Typical *T. equigenitalis* strains have three copies of the rRNA operon per genome [12]. The 95% detection limit of real-time PCR (0.77 fg/reaction) is equivalent to ~1.2 copies of the 16S rRNA gene per reaction, showing that its analytical sensitivity is high. On the other hand, cPCR2 and cPCR3 need at least 160 copies per reaction to detect *T. equigenitalis*.

![Fig. 1. Boxplots of 95% detection limits (fg/reaction). The boxplot gives information on minimums, maximums, 25th percentiles, 75th percentiles, and medians. Average values of five *Taylorella equigenitalis* strains are shown under the plots.](image-url)

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**Table 1.** Information of nucleic acid amplification tests used in this study

| Assay ID       | Target gene or locus tag | Reference |
|---------------|--------------------------|-----------|
| Conventional PCR1 | 16S rRNA                 | [6]       |
| Conventional PCR2 | 16S rRNA                 | [9]       |
| Conventional PCR3 | 16S rRNA                 | [3]       |
| Conventional PCR4 | 16S rRNA                 | [5]       |
| Real-time PCR   | 16S rRNA                 | [21]      |
| Semi-nested PCR | Hypothetical protein (EL142_RS00865)-Dephospho-CoA kinase (EL142_RS00870)† | [2]       |
| LAMP*           | 23S rRNA                 | [14]      |

*LAMP, loop-mediated isothermal amplification. †Accession number of NCTC11184 strain is NZ_LR134346.

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**Table 2.** Bacterial strains used in this study

| Bacterial species (strain name) | Number of strains |
|---------------------------------|-------------------|
| *Taylorella equigenitalis* (K188, CEMO20, CEMO37, Te80, and Te95†) | 5 |
| *Taylorella asinigenitalis* (ATCC 700933T and UK-1) | 2 |
| *Achromobacter xylosoxidans* (JCM 9659T) | 1 |
| *Alcaligenes faecalis* (JCM 20522T) | 1 |
| *Pusillimonas noettemanni* (DSM 10065T) | 1 |
| *Bordetella avium* | 1 |
| *Bordetella bronchiseptica* | 3 |
| *Pseudomonas aeruginosa*† | 3 |
| *Escherichia coli*† | 3 |
| *Streptococcus zooepidemicus*† | 3 |
| *Staphylococcus aureus*† | 3 |
| *Klebsiella pneumoniae*† | 3 |

*Members of family Alcaligenaceae. †Strain K188 was isolated from United States of America in 1997, CEMO20 was from Switzerland in 1988–89, and the others were from Japan between 1985–2000. ‡These species are frequently isolated from specimens of horses.
Next, we evaluated the validities of the seven NAATs against clinical specimens by comparison with bacterial isolation. To obtain clinical samples, we experimentally infected two Thoroughbred horses with *T. equigenitalis* strain (K188 or CEMO37) and collected 42 swabs from the genital tracts (e.g., clitoral sinus and fossa) over 2 weeks. Swabs were suspended in 500 µl of distilled water, and each 100 µl of suspension was used for DNA extraction and bacterial isolation. After extracting the genomic DNA in Instagene Matrix resin (Bio-Rad Laboratories), we conducted each NAAT. Bacteria were isolated on chocolate blood agar following a World Organisation for Animal Health (OIE) manual [18]. All samplings and experiments with infectious materials were conducted in accordance with ethical and welfare regulations of the Animal Care Committee of the Equine Research Institute under approval number 16-9.

From the 42 clinical samples, real-time PCR and snPCR obtained the most positives (33 out of the 42), followed by bacterial isolation (31), cPCR1 and LAMP (30), and cPCR4 (26) (Supplementary Table 3). On the other hand, cPCR2 and cPCR3 detected only two and seven positive results, respectively.

The analytical detection limits and the positive numbers in clinical samples varied greatly depending on the molecular assay used. Assays with better detection limits tended to identify more positive specimens, with some exceptions; e.g., the detection limit of snPCR (6.42 fg/reaction) was inferior to that of LAMP (0.89 fg/reaction), but snPCR identified more positives. The difference in the amounts of DNA templates used may have affected the results: 5 µl of DNA for snPCR and 2 µl for LAMP. In addition, as the clitoral sinus and fossa, which are the main sampling sites for CEM, are easily contaminated with feces and urine, the many substances such as bacterial proteases, nucleases, and bile acids in these contaminants could interfere with DNA amplification through physicochemical and enzymic effects [22]. Differences in the resistance of each assay to the inhibitors might have affected the test efficacies.

In comparison with bacterial isolation, only real-time PCR and snPCR gave superior results in terms of positive samples, and the other NAATs may not offer significant advantages in their use. In particular, low detection efficiencies of cPCR2 and cPCR3 could lead to large numbers of false-negative results. Therefore, we believe that the NAAT used to detect *T. equigenitalis* should be carefully selected; i.e., cPCR2 and cPCR3 would not be suitable for quarantine purposes.

Although real-time PCR and snPCR did not differ with the experimental samples, they might have different efficiencies in samples with low DNA concentrations. So we compared them further. We mixed several genital swab specimens to prepare 46 pooled suspensions, all of which were confirmed as negative for *T. equigenitalis* by real-time PCR and snPCR. Three strains of *T. equigenitalis* (K188, CEMO20, and CEMO37) were suspended separately in a saline solution and adjusted to an optical density of 0.1 at 600 nm (original sample). Ten-fold serial dilutions of the suspensions were prepared from the original solution to 1/100,000. We added 1 µl of each dilution to 79 µl of each pooled suspension, obtaining 46 spiked samples at each concentration of each *T. equigenitalis* strain (138 spiked samples in total at each concentration). Bacterial DNA was extracted by boiling the spiked suspensions at 100°C for 10 min. Real-time PCR and snPCR were conducted in two laboratories. Both laboratories used the same protocol for snPCR, but each used a unique real-time PCR probe (3′BHQ1 or 3′MGB) and instrument (StepOne Plus or QuantStudio 5; Thermo Fisher Scientific, Tokyo, Japan) for real-time PCR (Supplementary Table 1). Positive and negative controls were placed on each reaction plate to confirm that the tests were valid each time.

Both real-time PCR and snPCR yielded positive results for all 138 spiked original samples (Table 3). Real-time PCR gave positive results for all samples in both laboratories at 1/10 to 1/1,000, but snPCR missed some samples. The numbers of positive results decreased at 1/10,000 and 1/100,000 by both real-time PCR (122 and 43 positives, respectively) and snPCR (77 and 7). The higher number of positives in both dilution groups by real-time PCR indicates that real-time PCR would be a more reliable assay at low DNA concentrations.

To evaluate the clinical performance of NAATs, it is important to compare them by using controlled specimens under test conditions. Such comparison revealed that both the detection limit and clinical efficacy varied greatly among the seven NAATs.

| Dilution step | Relative bacterial concentration | Test   | Positive* | Intermediate* | Negative* |
|---------------|---------------------------------|--------|-----------|--------------|-----------|
| 0 (Original, OD600=0.1) | 1 | real-time PCR | 138 | 0 | 0 |
| | | snPCR | 138 | 0 | 0 |
| 1 | 1/10 | real-time PCR | 138 | 0 | 0 |
| | | snPCR | 136 | 2 | 0 |
| 2 | 1/100 | real-time PCR | 138 | 0 | 0 |
| | | snPCR | 134 | 4 | 0 |
| 3 | 1/1,000 | real-time PCR | 138 | 0 | 0 |
| | | snPCR | 131 | 3 | 4 |
| 4 | 1/10,000 | real-time PCR | 122 | 16 | 0 |
| | | snPCR | 77 | 54 | 7 |
| 5 | 1/100,000 | real-time PCR | 43 | 48 | 47 |
| | | snPCR | 7 | 49 | 82 |

*Positive, positives in both facilities; Intermediate, positive in one facility and negative in the other; Negative, negatives in both facilities.
Our results indicate that the selection of the appropriate NAAT is important for the efficient detection of *T. equigenitalis* in clinical samples. In particular, real-time PCR and snPCR were found to be superior to other NAATs and bacterial isolation to detect *T. equigenitalis* from experimental clinical specimens, and some cPCRs showed low efficacies. In situations where cPCR must be used, cPCR1 should be selected because it had the best performance among the four cPCRs here. In addition, real-time PCR was more sensitive than snPCR at low DNA concentrations, and thus would be the most reliable with the fewest false-negatives.

The few enzymes and DNA extraction methods, which affect both the detection sensitivity and resistance to crude specimens, compared here is a limitation of this study. For the use of different NAAT conditions, the efficiency of detecting *T. equigenitalis* should be confirmed beforehand according to purposes.

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