STANDARD OPERATING PROCEDURE

REAL-TIME PCRs FOR THE DETECTION OF TAYLORELLA EQUIGENITALIS

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This SOP is a method used at the EURL, all real time PCR based methods including commercial kits validated and used successfully in the PT can be used for this essay.

Extracts from the world organisation for animal health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals – Chapter 3.5.2, contagious equine metritis (version 2018):
"Molecular testing methods such as polymerase chain reaction (PCR) and real-time PCR are now commonly used to detect *Taylorella* both from swabs and culture plates. They have the advantage of speed of result and can easily differentiate between *T. equigenitalis* and *T. asinigenitalis*."

"The direct detection of *T. equigenitalis* by PCR has several advantages over isolation of the bacteria by culture. First, PCR is less vulnerable to contaminating flora, which reduces the number of false-negative results. Secondly, the turnaround time of the PCR is much shorter than the minimum 7-day culture time with isolation. And thirdly, as only DNA is detected rather than viable organisms, the need for rapid transport of specimens to the laboratory is reduced."

"A strict PCR regime to avoid DNA cross contamination should be deployed in diagnostic laboratories."

"A real-time PCR was developed in the UK for use directly on genital swabs and compared with culture (Wakeley et al., 2006[1]) and this has been subsequently used for pre-breeding screening studies (Ousey et al., 2009[2]). There was no significant difference in the performance of the direct PCR and culture, but the PCR had the added advantage of speed of result and also differentiated *T. equigenitalis* from *T. asinigenitalis*. Commercial PCR kits are available for the detection of *T. equigenitalis* and these may be used to enhance the testing capabilities of authorised laboratories."

1. COLLECTION OF SAMPLES

Concerning trade and imports in the European Union, the implementing regulation (EU) N ° 846/2014[3] [3] specifies:

- the specimens* shall be placed in transport medium with activated charcoal, such as Amies medium, before dispatch to the laboratory,
- PCR for the detection of genome of *T. equigenitalis*, carried out within 48 hours after taking the specimens from the donor animal.

* Specimens: penile sheath (prepuce), urethra and fossa glandis for stallion / mucosal surfaces of the clitoral fossa and clitoral sinuses for mare.

Apart from this context, the manufacturers of commercial PCR kits may have validated other types of swabs and transport conditions. Refer to the supplier's recommendations.

This method can also be applied directly on pure culture bacteria in case of second-line analysis to confirm a CEM case.

[1] WAKELEY P.R., ERRINGTON J., HANNON S., ROEST H.I.J., CARSON T. HUNT B. & HEATH P. (2006). Development of a real time PCR for the detection of *Taylorella equigenitalis* directly from genital swabs and discrimination from *T. asinigenitalis*. Vet. Microbiol., 118, 247–254.
[2] OUSEY J.C., PALMER L., CASH R.S.G., GRIMES K.J., FLETCHER A.P., BARRELET A., FOOTE A.K., MANNING F.M. & RICKETTS S.W. (2009) An investigation into the suitability of a commercial real-time PCR assay to screen for *Taylorella equigenitalis* in routine prebreeding equine genital swabs. Equine Vet. J., 41, 878–882
[3] COMMISSION IMPLEMENTING REGULATION (EU) No 846/2014 of 4 August 2014 amending Annex D to Council Directive 92/65/EEC as regards the conditions for donor animals of the equine species.

2. TECHNIQUE

The EURL validated and uses under COFRAC accreditation the Wakeley real-time PCR upgraded with the addition of an internal control of the DNA extraction and amplification (DNA Extraction Control 670 kit from Bioline).

The table below presents the different stages of its analytical process:

	SAMPLE	Swab	Bacterial strain
ANALYTICAL PROCESS	**Preparation**	Dip the swab 5 s in 0.2 ml of ultrapure nuclease-free water	Put one colony in 1 ml of ultrapure nuclease-free water and make an aliquot of 0.2 ml
		Add the DNA Extraction Control 670 according to the supplier's recommendations	
	Extraction	DNA extraction performed using the Macherey-Nagel NucleoSpin Tissue kit	Heat at 95–100°C for 15 min in a heat block
	Preparation of reagents and mix PCR	Prepare the Mix PCR	
		Distribute the Mix PCR according to plate plan	
		Add the DNA templates	
	Amplification	Run PCR	
	Analysis of results	Analyse the amplification signals	
		Express the results	

The table below presents the oligonucleotides used for the detection of *T. equigenitalis*:

Primer/Probe	Sequence (5'-3')	Concentration
Tay377for	CCGCGTGTGCGATTGA	10 µM
Tay488rev	TTTGCCGGTGCTTATTCTTCA	10 µM
TequiFAM	[6FAM]AAAGGTTTGTGTTAATACCATGGACTGCTGACGG[TAM]	10 µM

In order to avoid a loss of performance due to the addition of an internal control detection, the detection of *T. asinigenitalis* (specific probe: TasiniHEX [HEX/VIC]AAAGTTTTAGGATAATACCCTAGGATGCTGACGG[TAM]) is performed independently of the detection of *T. equigenitalis*.

In addition to the Wakeley real-time PCR, several commercial real-time PCR for the *T. equigenitalis* detection were validated by the EURL and listed below:

Commercial real-time PCR for the *Taylorella equigenitalis* detection	
cador *T. equigenitalis* PCR Kit	INDICAL BIOSCIENCE
ADIAVET™ CEMO TAYLORELLA REAL TIME (detection of *T. equigenitalis* and *T. asinigenitalis*) [a]	Bio-X Diagnostics / ADIAGENE
Taylorella equigenitalis DNA gyrase subunit B (*gyrB*) gene genesig® Advanced Kit (does not contain qPCR Master Mix) [a]	Primerdesign™ Ltd
cador TKP PCR Reagent (detection of *Taylorella equigenitalis* and *Klebsiella pneumonia, Pseudomonas aeruginosa*)	INDICAL BIOSCIENCE

[a] Does not target the 16S ribosomal DNA.

3. PREPARATION OF A POSITIVE CONTROL SAMPLE IN DETECTION LIMIT

In the absence of a characterized reference material, a positive control sample in detection limit may be produced to be used as positive control of the process. The production below is presented as an example:

- In sterile condition, prepare in PBS pH 7.2 several serial dilutions of reason 10 (or less) from colonies of a *T. equigenitalis* control strain,
- Dip eight sterile swabs in each dilution of the *T. equigenitalis* suspension and place the swabs in Amies-charcoal medium,
- Detect the presence of *T. equigenitalis* during two successive runs of PCR: one run with four swabs per dilution and a second run of PCR with the four remaining swabs per dilution. Take care the swabs as field swabs.

The positive sample in detection limit correspond to last dilution showing 100% of positive results. This dilution of the *T. equigenitalis* suspension will be aliquoted in single doses of 0.2 ml and stored at ≤ -16°C.

A positive control sample in detection limit could also be produced for *T. asinigenitalis*.

4. PREPARATION OF FIELD AND CONTROL SAMPLES

4.1. SWABS (EURL PROTOCOL TO BE ADAPTED ACCORDING TO THE PROTOCOL USED)

- Sample(s):
  - dip the field swab 5 s in 0.2 ml of ultrapure nuclease-free water,
  - add the DNA Extraction Control 670 according to the supplier's recommendations.
- Negative control process:
  - place one swab in Amies-charcoal medium,
  - take back the swab and dip it in 0.2 ml of ultrapure nuclease-free water,
  - add the DNA Extraction Control 670 according to the supplier's recommendations.
- Positive control process:
  - dip one sterile swab 5 s in 0.2 ml of positive control sample in detection limit,
  - place the swab in Amies-charcoal medium,
  - take back the swab and dip it in 0.2 ml of ultrapure nuclease-free water,
  - add the DNA Extraction Control 670 according to the supplier's recommendations.

Perform the DNA extraction using the Macherey-Nagel NucleoSpin Tissue kit according to the supplier's recommendations from each tube of sample(s), negative control process and positive control process.

4.2. BACTERIAL STRAINS (EURL PROTOCOL TO BE ADAPTED ACCORDING TO THE PROTOCOL USED)

In this case, the template is not the limiting factor so there is no point in using a positive control sample in detection limit. The positive control process can be bacterial lysate produced from a *T. equigenitalis* suspension heated at 95–100°C for 15 min in a heat block. This positive control could also be produced for *T. asinigenitalis*.

- Sample(s):
  - put one colony in 1 ml of ultrapure nuclease-free water,
  - make an aliquot of 0.2 ml,
  - add the DNA Extraction Control 670 according to the supplier's recommendations.
- Negative control process:
  - add the DNA Extraction Control 670 according to the supplier's recommendations in 0.2 ml of ultrapure nuclease-free water.
- Positive control process:
  - put one colony of *T. equigenitalis* control strain in 1 ml of ultrapure nuclease-free water,
  - make an aliquot of 0.2 ml,
  - add the DNA Extraction Control 670 according to the supplier's recommendations.

Perform the DNA extraction by heating at 95–100°C for 15 min in a heat block each tube of sample(s), negative control process and positive control process.

5. VALIDATION AND INTERPRETATION OF RESULTS

5.1. VALIDATION OF THE ANALYTICAL PROCESS

The validation of the analysis is carried out by observing the fluorescence curves generated from the control samples and the internal controls.

When the fluorescence values are plotted on a logarithmic scale, the operator fixes

- the baseline which must be positioned as low as possible without including the background noise but high enough to cross the non-characteristic part of the fluorescence curves of the positive controls obtained during the first cycles,
- the threshold line (= minimum fluorescence limit to be reached for a fluorescence signal emitted to be significantly greater than the background noise signal represented by the baseline) which must be crossed the middle of the curves of fluorescence of positive controls during the exponential increase phase of amplification.

For each control, the operator observes the presence or absence of an amplification signal and checks the consistency of this signal in order to validate or invalidate the controls (absence of a characteristic amplification curve for the negative controls and presence of a characteristic amplification curve for the positive controls and internal controls).

If the analysis series is validated, the operator observes the presence or absence of an amplification signal and checks the consistency of this signal for all the samples.

If the series of analysis is not validated, and depending on the anomaly observed, all or part of the analytical process has to be redone. In the presence of an uncharacteristic (flattened) amplification curve from both sample and internal control, the sample can be analysed again after dilution of the DNA extraction to 1:10 and/or after carrying out a new DNA extraction from the initial sample.

5.2. INTERPRETATION OF RESULTS

For a swab matrix sample, the result can be expressed as follows:

- “Detected” when the Ct value is ≤ Ct value of the positive control in detection limit and the signal corresponds to a characteristic amplification curve,
- “Detected in detection limit” when the Ct value is > Ct value of the positive control in detection limit and the signal corresponds to a characteristic amplification curve,
- “Not detect” in absence of amplification signal,
- “Uninterpretable or not conclusive” when one or more signal analysis criteria do not match the expected characteristics.

For a bacterial strain matrix sample, the interpretation “Detected in detection limit” is not applicable.

6. STORAGE CONDITIONS AND ELIMINATION OF SAMPLES

Store products and consumables in keeping with suppliers’ recommendations.
Keep all control strains (e.g. cryobeads) in a deep-freezer at ≤ - 65°C.
Keep all swab samples in a refrigerated unit at (5 ± 3)°C or a freezer at ≤ - 16°C until the results are sent.

The cell lysates, DNA extractions and PCR products may be kept in a freezer at ≤ - 16°C.