10 years of *Taylorella equigenitalis* ring trial results comparing culture and polymerase chain reaction

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Summary

Taylorella equigenitalis, the cause of contagious equine metritis (CEM), can be detected by culture but in recent years polymerase chain reaction (PCR) has also been used. In 2008, the World Organisation for Animal Health (OIE) reference laboratory in the United Kingdom (UK) set up a ring trial for laboratories to assess their ability to identify *T. equigenitalis* in laboratory prepared samples because the identification of *T. equigenitalis* in the laboratory was recognised to be difficult. Freeze-dried culture suspensions in various combinations of any of *T. equigenitalis*, *Taylorella asinigenitalis*, other typical equine contaminant organisms, or no organism were used. All laboratories provided culture results and some also gave PCR results. The results reported here cover the 10 years since inception and look at the ability to identify *T. equigenitalis* under ideal laboratory conditions, a fundamental necessity to be able to detect its presence in equine genital samples. The detection rate was very high by both methods. The accuracy was not significantly different between the culture and PCR methods for pure *T. equigenitalis*

samples. For *T. equigenitalis* mixed with contaminants, culture missed about 2% ($p = 0.02$) compared with PCR, which was over 99% accurate. Difficulty in differentiating *T. asinigenitalis* from *T. equigenitalis* was apparent in a number of laboratories for both culture and PCR in 2008 but was less evident in 2016.

It was also noted that culture results from laboratories that also tested by PCR had around 4% higher detection rates ($p < 0.05$) of *T. equigenitalis* than those that only used culture.

Keywords

Contagious equine metritis – Equine – Horse – *Taylorella equigenitalis* – Test performance.

Introduction

Testing for *Taylorella equigenitalis*, the contagious equine metritis organism (CEMO), traditionally uses culture according to the World Organisation for Animal Health (OIE) *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, although polymerase chain reaction (PCR) tests are also available (1) and more recently quantitative PCRs (qPCRs) have been developed and are now widely used (2, 3, 4). The annual ring trials reported here were originally organised by the OIE reference laboratory at the Animal and Plant Health Agency (APHA), Bury St Edmunds, United Kingdom (UK), and samples were prepared and distributed blind via the Vetqas division of APHA that specialises in international proficiency schemes for test performance.

The object of the ring trial was to provide laboratories with an opportunity to participate in external quality assurance to assess their proficiency in identifying *T. equigenitalis* using their selected laboratory test method for CEMO. After some years of running the ring trial it became apparent that the data could be used to summarise the performance of culture and PCR in a statistically valid way.

Materials and methods

The ring trials

A list of laboratories known to be testing samples for *T. equigenitalis* was compiled, including, where the information was available, national reference laboratories, and invitations were sent each year requesting their participation. The list was updated with the addition of new participants or removal of non-participants from time to time. Participation was free of charge; the funding was provided by the UK Department for Environment, Food and Rural Affairs (Defra) via a project run by the Veterinary Laboratories Agency (VLA, now named APHA), under its remit as an OIE reference laboratory for contagious equine metritis (CEM).

Each year since 2008 a panel of samples has been sent out and tested by participating laboratories for the presence of *T. equigenitalis* by culture and/or PCR at their own choice. There were some common participants in all years and other laboratories participated inconsistently. The samples contained freeze-dried culture suspensions in various combinations of any of *T. equigenitalis*, *Taylorella asinigenitalis*, other typical equine contaminant organisms, or no organism at all. The sample provenances are divided into five categories for analysis.

The bacterial load of each panel sample was sufficiently high to ensure that if *T. equigenitalis* were present it would grow in high numbers on culture using normally recommended methods described in the OIE *Terrestrial Manual* (1), so several log colony forming units per millilitre (CFU/ml) were present in each sample vial. Each ring trial panel sample comprised a vial of the bacterial suspension dispensed in a standard volume and freeze-dried so that all vials of each sample were uniform in content. The vial was reconstituted with broth in a prescribed manner. Vetqas, the APHA's independent proficiency test provider, prepared the samples, distributed them and collected results via its usual process. They provided the raw test results anonymously in Excel spreadsheets to the author, who substantially reorganised the data for statistical analysis.

The culture or PCR method used by each laboratory was not routinely reported in the ring trial return, but in 2017 some incomplete data were obtained on whether the PCR was quantitative or not. The PCR, when used, was usually done directly on the sample and was sometimes used to confirm identification of an organism cultured from the sample. In this analysis a reported PCR test result is a direct test on the sample.

If a laboratory recorded *T. equigenitalis* 'suspected' as their result these were classified as *T. equigenitalis* positive because they indicated a belief that *T. equigenitalis* was present and were clearly not classed as *T. equigenitalis* negative. 'Inconclusive' results, on the other hand, were classed as *T. equigenitalis* negative because they did not clearly indicate belief that the sample contained *T. equigenitalis*. For reasons that are unclear, a result for some samples was not reported by a participant laboratory, sometimes for one test but occasionally for both tests. These were analysed as missing values.

Analysis

The object of this study was to assess the performance of culture and PCR in identifying *T. equigenitalis* in a given sample across a number of different laboratories. Each individual sample result was treated as independent for the purposes of statistical analysis unless otherwise stated. Statistically the laboratories are independent. Although many participating laboratories took part in repeated years it was considered reasonable to treat each year as independent, and although the same samples were tested by a number of laboratories in each year it was deemed reasonable to treat these as independent, because any correlation within samples and within laboratories was unlikely to be important in the overall assessment. It is not known why some results were missing and it may be a source of bias in the results if the reason for a nil return was because the laboratory was uncertain of the accuracy of their result and therefore decided not to disclose it. Possible factors associated with missing values were examined.

Analyses were stratified by the five sample categories (Tables I and II) to identify whether there was an influence of sample

provenance. Comparison of test performance between PCR and culture was only done on the paired results for the same sample from the same laboratory. These were analysed by McNemar's test, and statistical significance for agreement between PCR and culture was calculated per sample category. The percentage accuracy was calculated as the percentage value of the number of correct results divided by the total number of results reported; hence for samples containing *T. equigenitalis* accuracy is the correct detection of *T. equigenitalis* and for samples not containing *T. equigenitalis* accuracy is the correct reporting of the absence of *T. equigenitalis*, i.e. *T. equigenitalis* negative. In 2008 and 2016 a sample containing *T. asinigenitalis* was included as a pure culture. The format of the return for culture in 2008 and 2016 was slightly different, which affected the way culture results were recorded (but not the PCR results). In 2008, culture and PCR results were reported as positive or not for *T. equigenitalis*. In 2016, the culture results were reported as *Taylorella* detected or not, which in the case of *T. asinigenitalis* samples was an ambiguous question. The *T. asinigenitalis* data were thus analysed separately from the results in the tables.

A comparison was made of culture performance between laboratories that use only culture and laboratories that use both tests, stratified by sample provenance, using a chi-squared or Fisher's exact test.

Statistical significance uses $p = 0.05$ for convenience. The statistical analysis used Stata14 software (Stata Corp, College Station, TX, United States of America [USA]).

Results

Overall, 54 different laboratories took part over the years, and between 25 and 41 laboratories took part each year. The results are considered in two parts: first, for those laboratories where culture and PCR were used in parallel so a comparison of culture and PCR could be made on an equivalent basis. Second, the performance of culture was compared between laboratories that did and did not also test using PCR.

Combining the 2008–2017 results, the overall accuracy of PCR and culture is shown in Table I, based on the correct identification of the presence or absence of *T. equigenitalis*. The sample categories were:

a) *T. equigenitalis* in pure culture condition

b) '*T. equigenitalis* with contaminant' samples contained *T. equigenitalis* with one or more other organisms such as *Escherichia coli*, *Streptococcus* spp., *Proteus* spp., *Enterobacter* spp. or *Staphylococcus* spp., which varied from sample to sample

c) 'contaminants only' samples contained one or more of these organisms and/or *Oligella* spp.

d) 'sterile' samples contained no organism at all.

Table I summarises the number of tests with concordant or discordant positive or negative results by each test method (culture and PCR), bearing in mind that only *T. equigenitalis*-containing samples are true positives. For example, for the contaminants-only sample type, 278 results were found negative by both tests, and 11 were recorded as positive for *T. equigenitalis*. However, a further 11 that were found to be culture negative were reported as PCR positive, and a further 7 that were found to be culture positive for *T. equigenitalis* were PCR negative. Significant differences between culture and PCR accuracy were found for *T. equigenitalis* with contaminants ($p = 0.02$) but not for the other sample categories.

Table I

Comparison of detection of *Taylorella equigenitalis* by culture and polymerase chain reaction

The PCR results are reported as either positive or negative for culture positive or negative samples, stratified by sample content, for paired culture and PCR results only. Positive means that *T. equigenitalis* has been reported as detected in the sample. Overall accuracy of each test is relative to the presence or not of *T. equigenitalis.*

Sample type	Number of samples	Years used	Number of sample results	Number of PCR results reported positive or negative for *T. equigenitalis* when culture is reported positive or negative				Accuracy % (95% CI)	
				Culture negative		Culture positive			
				PCR Neg	PCR Pos	PCR Neg	PCR Pos	Culture	PCR
Contaminants only	16	10	307	278	11	7	11	94.1 (90.9–96.5)	92.8 (89.3–95.4)
Sterile	8	6	143	134	4	1	4	96.5 (92.0–98.9)	94.4 (89.3–97.6)
T. equigenitalis pure	9	5	187	0	0	1	186	100 (98.0–100)	99.5 (97.1–100)
T. equigenitalis with contaminants	16	8	315	1	7	0	307[a]	97.5 (95.1–98.9)	99.7 (98.2–100)

a: $p = 0.02$
CI: confidence interval
Neg: negative
PCR: polymerase chain reaction
Pos: positive

For *T. asinigenitalis*, of the 19 returns with both culture and PCR results in 2008, 2 were classified by both tests as positive for *T. equigenitalis* and 8 as negative; 6 were culture positive and PCR negative; and 3 were culture negative and PCR positive. There was no difference in the overall accuracy of the two tests ($p = 0.5$). For culture there were thus 11 negatives and 8 positives for *T. equigenitalis* in laboratories also using PCR, whilst in laboratories that did not use or report PCR, there were 5 negatives and 12 positives by culture; this was not a significant difference ($p = 0.1$). Overall, six culture results did specifically identify *T. asinigenitalis*. Two of these did not test by PCR, but four did and two were PCR positive for *T. equigenitalis*. In 2016, when the culture results were reported only as *Taylorella* detected or not, the results were hard to classify relative to the presence or not of *T. equigenitalis*. Of the 31 returns, 2 had missing culture results, 3 found no *Taylorella*, 20 laboratories specified that *T. asinigenitalis* had been cultured and 16 were also tested by PCR. The PCR results were clearly reported as *T. equigenitalis* positive or negative. Overall, there were 20 samples PCR negative and 1 PCR positive for *T. equigenitalis*. The single PCR positive erroneously identified as *T. equigenitalis* had a *Taylorella* positive culture result but the species was not specified.

Table II shows the performance of culture when the results are split by whether a PCR result was also reported on the same sample or not. There were statistically significant differences in the ability of culture to find *T. equigenitalis*, with the culture-only laboratories failing to identify the organism more often than laboratories parallel testing by PCR; the difference was 4.4% for pure *T. equigenitalis* and 3.7% for *T. equigenitalis* with contaminants.

Table II

Comparison of the accuracy (percentage of tests correct for presence or absence of *T. equigenitalis*), with 95% confidence intervals, of culture results split by whether the laboratory also tested samples by polymerase chain reaction or not

	Culture results by laboratories co-testing with PCR or not			
	With PCR	No PCR	With PCR	No PCR
Sample type	*n*	*n*	Accuracy % (95% CI)	Accuracy % (95% CI)
Contaminants only	307	184	94.1 (90.9–96.5)	94.0 (89.6–97.0)
Sterile	143	94	96.5 (92.0–98.9)	99 (94.0–100)
T. equigenitalis pure	187	113	100 (98.0–100)	95.6[a] (90.0–98.5)
T. equigenitalis with contaminants	315	162	97.5 (95.1–98.9)	93.8[b] (88.9–97.0)

a: $p < 0.01$
b: $p = 0.05$
n: number tested
CI: confidence interval
PCR: polymerase chain reaction

'Suspicious' was used for four culture results and 'inconclusive' for one culture and one PCR result in total. The term 'suspicious of *T. equigenitalis*' was used by one laboratory for all their samples that were not recorded as negative in that year, and it was clear they had identified *Taylorella*. 'Inconclusive' was used for one culture and one PCR result when other *T. equigenitalis* positive samples in those laboratories had been clearly entered as positive. In all, only five culture results and one PCR result were thus reclassified to positive or negative to allow them to be included in the analysis.

The proportion of laboratories using PCR over the 10-year period was between 62 and 82% with a mean of 69%. This did not show any pattern of increasing or decreasing use over the years. Some laboratories used PCR in early years then ceased, others took it up later and some used PCR in occasional years, but in most cases if a laboratory used PCR it continued through all the years in which it participated in the ring trials. It was not possible from the data provided to make a detailed observation on whether the PCR type changed over the years, owing to a lack of consistent information in the returns. By 2017, however, it was clear that qPCRs were used most commonly but not exclusively.

There were a relatively large number of missing values for culture. Inspection of the data indicates that the vast majority of these (n = 41) are due to nil returns for the whole panel by a given laboratory in a given year, even though in some cases PCR results had been fully reported. There were 11 sporadic missing culture values, and 12 missing PCR results in those laboratories doing a PCR return in that year. These were prior to 2014, and six were when no growth was recorded on culture, but there were also missing values for samples with contaminant growth and when *T. asinigenitalis* or *T. equigenitalis* was found on culture.

Discussion

The results in laboratories that ran both tests indicate that there is little absolute difference in the ability of PCR and culture to detect *T. equigenitalis* and that the detection rates are very high. Since the ring trial panels are not representative of the incidence of such sample types in the field it is not appropriate to talk about test sensitivity or specificity but the accuracy of detection, knowing that the samples' true status can be split into the different sample provenance categories. Pure *T. equigenitalis* samples were routinely detected. When *T. equigenitalis* was mixed with another organism the detection rate of culture diminished slightly, at 97.5% compared to 99.7% for PCR, and this difference was statistically significant (p = 0.02). In many of these cases the laboratory would be confirming their culture

identification by PCR. Failure to find a positive sample by culture in this instance is therefore most likely due to a *T. equigenitalis* culture colony not being seen on the plate or the wrong colony being selected for confirmatory PCR testing. The slightly better detection of *T. equigenitalis* by PCR than culture in similar circumstances, but using laboratory-prepared swabs as the sample type, was noted by Petry *et al.* (4), who recently published data on two European Union (EU) ring trials in which details on the PCR and culture methods were also examined. Studies using field samples indicate that parallel testing of swabs by culture and PCR gives either similar results or higher detection rates for PCR (2, 3, 5, 6, 7).

The inclusion of *T. asinigenitalis* in the panels caused some predictable errors when it was confused with *T. equigenitalis*. This was more apparent in 2008, for both culture and PCR, than in 2016. In 2016 only one false positive PCR result was recorded, 20 of the 26 *Taylorella* positive results for culture specifically identified that they had cultured *T. asinigenitalis*, and no one specified that they had identified it as *T. equigenitalis*. In 2008, qPCRs which differentiated *T. equigenitalis* and *T. asinigenitalis* had only recently been published (2), and it is likely that many non-differentiating conventional PCRs were in use in the 2008 ring trial, whilst in 2016 most of the PCRs used were described as quantitative and the most commonly used is able to differentiate *T. asinigenitalis* from *T. equigenitalis* (2).

It is assumed that samples not tested by PCR were reported by laboratories that do not have PCR available, thus implying that any difference in the accuracy of culture between culture-only testing and culture-and-PCR parallel testing is actually a difference between the two types of laboratory. The accuracy of a culture result in these two types of laboratory indicated no significant difference for non-*T. equigenitalis* containing samples, as shown in Table II. However, for both sample types containing *T. equigenitalis* (pure and with contaminants), there was a statistically significant difference in the ability of culture to find *T. equigenitalis*, with the culture-only laboratories failing to identify the organism more often than laboratories parallel testing by PCR, the difference being around 4%.

The reason for this difference in culture accuracy is not explained by the results but it may be due to the ability of the laboratory to select *T. equigenitalis* colonies from the plate for speciation testing in mixed cultures, if selective media for *T. equigenitalis* have not been very effective, or a possible improvement in correct identification of colonies learned by conducting a confirmatory PCR on selected colonies. However, this would not explain the failure to identify pure *T. equigenitalis* samples.

In both Tables I and II, there was a low but noticeable number of false positives with both tests in samples with no *Taylorella* spp. present. This is likely to reflect occasional cross-contamination between samples. There is a hint, but no statistically significant differences, that PCR may produce more false positives than culture. The lack of detail on the PCR method used prevented investigation of whether this is more likely with conventional or qPCR.

Various reasons may account for nil returns, including practical processing problems and reluctance to report any result due to lack of confidence in some results in the panel, but it is not possible to interpret the missing results any further than this and it is not an uncommon observation in ring trial distributions.

The findings of the ring trial analysis can only be partially extrapolated to the accuracy of testing in field situations. The samples provided were culture suspensions, so they do not contain the full genital flora or the equine detritus that would be contained in a field sample; nor do they show the effect of the swab itself (typically maintained in Amies transport medium). The pure *T. equigenitalis* samples show the fundamental ability of a laboratory to grow a single organism and identify whether it is or is not *T. equigenitalis*. The added contaminants seem to slightly reduce the ability to identify *T. equigenitalis* by culture, possibly because contaminants can overgrow the *T. equigenitalis* colonies or because colony selection for confirmatory testing is not 100% accurate. Direct PCR on swabs does not require colony selection and this avoids the need for this skilled step.

There were some false positives in contaminant-only or sterile samples by both culture and PCR. For PCR at least, this would most likely indicate contamination of the sample with *T. equigenitalis* either from positive control material or from other samples in the panel. Alternatively, it may indicate a lack of specificity of some of the PCRs used. This was not a validation study and the individual PCRs used were not generally declared in any detail.

In descriptions of PCR validation (2, 3, 5) there is a quantitative determination that usually indicates the CFU per sample that the PCR is able to detect. This CFU level is calculated from dilutions of a known concentrated sample and is not the same as, and not to be confused with, the CFU level that can be detected by direct culture on the same sample. Indeed, there is very little published information, to the author's knowledge, on the detection limit in CFU of culture even in pure laboratory-prepared samples, although Ousey *et al.* (3) indicated a similar CFU detection level in pure culture suspensions between qPCR and culture. The ability to detect a low number of colonies by culture is affected not only by dilution but also by the masking or overgrowth of *T. equigenitalis* colonies by other organisms on the plate, which in field samples can be extensive and not fully inhibited by addition of antibiotics or the use of selective growth media. In the ring trial samples used here there is always an adequate amount of the organism present to be easily found; the samples provided are not expected to give a scant growth of *T. equigenitalis.*

Conclusions

The ring trials show the fundamental ability of a laboratory to detect *T. equigenitalis* under favourable conditions with good sample quality and no other confounding factors that arise in field samples. Overall PCR was marginally but significantly more likely to detect *T. equigenitalis* than culture.

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