Validation of an Easy Handling Sample Preparation and Triplex Real Time PCR for Rapid Detection of T. equigenitalis and Other Organisms Associated with Endometritis in Mares

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Abstract

Isolation and identification of *Taylorella equigenitalis*, the causative agent of contagious equine metritis (CEM), by bacteriology is laborious and does not permit differentiation from the other member of the genus, *Taylorella asinigenitalis.* Moreover, other organisms such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* can also cause endometritis in mares and warrant diagnostic detection. Our objectives were to develop a rapid preparation method for field swab samples and to validate this protocol using new multiplex real time PCR (rtPCR) detection tools for identification of these four pathogens. The complete analytical process from sample preparation to PCR analysis was then evaluated against bacteriology, the World Organisation for Health's (OIE) gold standard method for *T. equigenitalis* and commonly used for the other three pathogens. The diagnostic sensitivity and specificity of this method, which used direct lysis and a multiplex rtPCR, were 100% and greater than 92% respectively. This study provided a simple-to-use method for pre-breeding screening of mares and stallions.

Keywords: Equine; Contagious Equine Metritis; direct lysis; rtPCR; diagnostics

1. Introduction

Bacterial endometritis is an important cause of subfertility in the mare. Endometrial infections are reported in 25-60% mares which failed to conceive following breeding, which contributes towards major economic losses for the equine industry [1]. Infection with *Taylorella equigenitalis* (*T. equigenitalis*) during natural or artificial breeding with contaminated semen from a carrier stallion [2,3] causes the disease contagious equine metritis (CEM), which results in a transient endometritis causing early embryonal death. *Taylorella equigenitalis* shares 98% sequence similarity with the other member of the *Taylorella* genus, *Taylorella asinigenitalis* (*T. asinigenitalis*), which was first isolated from donkey jacks in the United States (US) in 1987 [4,5]. *Taylorella asinigenitalis* is considered non-pathogenic in horses [4-7] and the main importance of this organism is the difficulty in differentiating it from *T. equigenitalis* using traditional bacterial culture [5,8]. Other opportunistic bacteria, especially *Klebsiella pneumoniae* (*K. pneumoniae*) and *Pseudomonas aeruginosa* (*P. aeruginosa*), are also venereally-transmitted pathogens in horses causing endometritis and infertility in mares [9,10].

Isolation and identification of *T. equigenitalis* by culture is recognized as the OIE gold standard for detection of the organism and is used for purposes of international trade. However, bacterial culture has several limitations including an extended period to obtain visible colonies (3-10 days), fastidious growth requirements (eg. microaerophilic conditions, selective media), overgrowth by other commensal flora from the urogenital tract, the possibility of false negatives due to low bacterial numbers in the sample [11,12], and the inability to distinguish colonies from *T. asinigenitalis* [13]. Various PCR methods have been developed to detect *T. equigenitalis* [11, 13-17] where sensitivity and/or specificity varied from 83.3 % to 100 % and offer good alternatives to culture. The objectives of this study were to develop an easy sample preparation method and to evaluate the performance of two rapid

and robust triplex rtPCRs, which allowed the detection and discrimination of *T. equigenitalis* from *T. asinigenitalis* and *K. pneumoniae* from *P. aeruginosa*, respectively.

2. Material and methods

2.1. Samples

Bacterial strains were used to evaluate the specificity and sensitivity of two rtPCRs and were obtained from the Institute Pasteur (Institut Pasteur, Paris, France), the American Type Collection (ATCC, Virginia, USA) or from the ANSES collection (ANSES Dozulé, EU reference laboratory, France) as described in table S1. Nucleic acid extracts from other bacteria and viruses used in this study were provided by ADIAGENE (ADIAGENE, Ploufragan, France) or isolated as described below in culture session, and were identified with VITEK® 2 (BioMérieux, Marcy L'étoile, France) or Maldi-Tof technologies (Bruker Daltonics, Wissembourg, France) at LABÉO (Supplementary data: table S1).

Genital swab samples (n=467; 288 from mares and 179 from stallions) were used to evaluate diagnostic specificity (Dsp) and sensitivity (Dse) and were obtained from 290 horses (1 to 5 swabs/animal) and 33 semen samples submitted to the LABÉO's (Caen, France) diagnostic CEM laboratory. Genital swabs samples (n=467) included clitoral sinuses/fossa (n=215), urethral fossa (n=75), cervix (n=73), urethra (n=51), penile sheath (n=30), urethral sinus (n=8), prepuce (n=9) and fossa glandis (n=6).

To obtain spiked samples, genital swabs collected from horses and confirmed negative for all four pathogens on both PCR and culture were vortexed in PBS and pooled. This negative solution was divided in 4 and spiked with 3 different level concentrations of each *T. equigenitalis* (CIP 7944) and *T. asinigenitalis* (CIP 107673) cultures. The titration of this solution was done on chocolate blood agar. One solution was kept as a control. Forty dry swabs (Copan, Brescia, Italy) were then dipped in the spiked solution and 6 dry swabs were

dipped in the control solution. All 46 swabs were kept at room temperature in a culture transport system (Copan) and subsequently sent for PCR analysis at ADIAGENE and LABÉO laboratories.

2.2. Culture conditions

Test swabs (467 clinical and 46 spiked samples) were analyzed within 24 hours (or 48 hours if transported under cold conditions) of preparation according to the OIE manual [18] and the French AFNOR Norm recommendations [19] for *T.* equigenitalis. Each swab was inoculated on 5% heated blood "chocolate" agar and *Taylorella* selective medium containing trimethoprim (1 µg/ml), clindamycin (5 µg/ml) and amphotericin b (15 µg/ml). Plates were incubated at 35-37°C, in 5-10% CO_2 microaerophilic condition at for least 7 days. The swabs were also streaked on Columbia blood agar supplemented with 5% sheep EDTA blood and eosin methylene blue agar (37°C +/- 2°C for 24-48 h) for the detection of *Pseudomonas* and *Klebsiella* bacterial species. Identification of the four pathogens was confirmed using Gram's test followed by VITEK® 2 (BioMérieux) or MALDI-TOF (Bruker Daltonics).

2.3. DNA extraction

Genomic bacterial DNA was extracted from 1ml of bacterial liquid culture (adjusted to 3 Mac Farland (McF)) using the QIAamp® DNA Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instruction and stored at -20°C (short term storage) or -80°C (long term storage). The DNA concentration of each extract was measured with a NanoDrop 2000c Spectrophotometer (Thermoscientific, Illkirch, France).

Swabs were vortexed in 500 µl of PBS, 100 µl was used for the direct lysis method (incubated 10 min at 95°C and cooled at room temperature or at +5°C+/- 3°C) and 200 µl was

used to perform a commercial column purification using QIAamp® DNA mini kit (Qiagen). DNA from 200 µl of semen was extracted with QIAamp® DNA mini kit (Qiagen).

2.4. PCR

Primers and probes were designed using the Primer Express Software V.3.0 (Life Technologies, Saint Aubin, France) to target *TEQUI_1010* (O-antigen export system permease protein RfbD, 1052325-1052410)*, TASI_0169* (Zinc ABC transporter, ATP-binding protein ZnuC, 171816-172112)*, ecfx* (extracytoplasmic function gene, GenBank accession no. DQ996551) and *mdh* (malate dehydrogenase housekeeping gene, GenBank accession no. AM051124) genes of *T. equigenitalis* [20], *T. asinigenitalis* [20], *P.* aeruginosa [21] and *K. pneumonia* [22], respectively. Two commercial PCR assays, ADIAVET CEMO *Taylorella* REAL TIME and ADIAVET KLEB/Pseudo REAL TIME (ADIAGENE) have been developed based on these genes.

For each experiment, 20 µl of amplification solution (ADIAGENE) was mixed with 5 µl of DNA extract. rtPCR was performed on a QuantStudioTM cycler (Life Technologies) using a program with initial steps at 50°C for 2 min and 95°C for 10 min followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Data were analyzed using the QuantStudioTM software (Life Technologies). Each assay included a negative control for the extraction step and negative (Rnase free water) and positive (calibrated DNA extracts) controls for the amplification steps.

Characterization of both ADIAVET REAL TIME PCR (ADIAGENE) (i.e. efficiency, repeatability, reproducibility, specificity and limit of detection (LOD)) and the complete analytical method (from DNA extraction to rtPCR analysis) were done according to the French AFNOR Norm [23]. To evaluate specificity, 24 different stains of *T. equigenitalis*, 14 of *T asinigenitalis*, 33 of *K. pneumoniae* and 16 of *P. aeruginosa* were used. The LOD_{Method}

was determined using 3 five-fold serial dilutions of calibrated bacterial suspension added to a negative emulsion of swabs or semen. Four replicates of each sample were extracted and amplified during 2 independent runs. The LOD_{Method} at 95% was the last concentration where 8 out of the 8 replicates were positive. The Dse and Dsp were determined by analyzing 500 clinical samples using rtPCR and comparing these results with the gold standard culture. The 95% score confidence interval for sensitivity and specificity was calculated using the Wilson method (EP12-A2 guide, 2008).

2.5. rtPCR inter-laboratory comparison assay

T. equigenitalis spiked swabs (3 levels of infection: high (480 LOD_{Method}), intermediate (48 LOD_{Method}), and low (4.8 LOD_{Method})) and T. *asinigenitalis* spiked swabs (3 levels of infection: 100, 10 and 1 LOD_{Method}) were used for the inter-laboratory comparison assay. A total of 46 swabs were sent to each laboratory (ADIAGENE and LABÉO), including 2 high, 8 intermediate and 10 low spike swabs for each pathogen and 6 negative controls. All analyses were conducted under blinded condition.

2.6. Analysis of data

For the determination of Dse and Dsp, statistical analyses were performed using Microsoft Excel 2010.The multifactorial ANOVA was performed with Sta51tGraphics® Centurion XVI Version 16.1.12 for windows (StatPoint Technologies Inc).

3. Results

All results regarding the characterization of the rtPCR and method are detailed in Table 1. A R^2 equal to 0.99 demonstrates a high degree of precision with a coefficient of variation (CV) <10%. No cross hybridization was observed with any of the bacteria or viruses tested

(Table S1). The Dse (calculated from the clinical samples) for *K. pneumoniae* and *P. aeruginosa* was 100% after both direct lysis and column purification methods, but could not be determined for *T. equigenitalis* and *T. asinigenitalis* as all tested clinical samples were found to be negative on both culture and rtPCR. The Dsp varied between 92.7% and 100% after direct lysis or column purification for all four pathogens (Table 1).

The results of the inter-laboratory comparison assay for the *Taylorella* species are illustrated in Figure 1A. For high (480 and 100 LOD_{Method} for *T. equigenitalis* and *T. asinigenitalis*, respectively) and intermediate (48 and 10 LOD_{Method} for *T. equigenitalis* and *T. asinigenitalis*, respectively) levels of spiking, all results obtained by direct lysis and column purification methods were classed as positive by both ADIAGENE and LABÉO. For low (4.8 and 1 LOD_{Method} for *T. equigenitalis* and *T. asinigenitalis*, respectively) spiking levels: 1) after direct lysis, 80% of *T. equigenitalis* and 90% of *T. asinigenitalis* spiked swab were found positive by both laboratories; 2) after column purification, only 50% of *T. equigenitalis* and 30% of *T. asinigenitalis* spiked swabs were found positive. All negative samples were found negative by both laboratories. The multifactorial ANOVA of cycle threshold (Ct) values obtained after analysis of *T. equigenitalis* samples (Figure 1B) reported significant differences between the levels of spiking (p-value <0.0001), the methods of purification (i.e. direct lysis or column purification, p-value < 0.0001) and the significant but limited difference between laboratories (p-value = 0.017). The multifactorial analysis of *T. asinigenitalis* samples reveals significant differences between the spiking levels (p-value <0.0001) and the purification methods (p-value = 0.0001), but no differences between the two laboratories (p-value = 0.29).

4. Discussion

The combination of direct lysis and rtPCR analysis offers a new, rapid screening tool for identification of *T. equigenitalis* and other organisms such as *T. asinigenitalis*,

K. pneumoniae and *P. aeruginosa* in both genital swabs and semen. Previously described PCR assays targeted the 16S rRNA gene [11], which may result in cross-reactivity with other bacteria such as *Oligella urethralis*. In order to increase the specificity of the amplification process, our approach targeted specific genes for each pathogen of interest. Using several different strains for each targeted pathogen the impact of genetic variability was evaluated and found to be negligible. Our results also demonstrated the absence of cross reactivity (i.e. exclusivity) of the assays with 91 unrelated pathogens, including *Oligella urethralis*. Evaluation of efficiency, repeatability and reproducibility verified the robustness of our rtPCRs. The LOD_{PCR} of our assays seemed higher than those calculated with the only commercially available kit [11]. The duration of the preparation process (i.e. direct lysis) for detection of *K. pneumoniae* and *P. aeruginosa* using the new method was greatly reduced when compared with column purification and with substantially better Dse and Dsp. Direct lysis has previously been proposed [11, 14] as an alternative to DNA purification, however the new protocol described here is significantly quicker (10 min vs 1 h) than previously reported [7, 10-15]. Regarding the characterization of the method, the Dse and Dsp obtained in our study (including the 4 pathogens) are comparable for both direct lysis and column purification to those obtained previously [11]. Due to the absence of positive field samples available in France, the Dse for *T. equigenitalis* and *T. asinigenitalis* was not calculated. As an alternative, a PCR blinded inter-laboratory comparison assay (n=46) for direct lysis and column purification was performed. As the limit of detection of the method was reach, increasingly discordant results were observed due to problems of reproducibility. These two rtPCR assays have been successfully used to test samples received in the context of official inter-laboratory comparison culture and rtPCR assays organized by the Horserace Betting Levy Board and the EU reference laboratory for equine diseases (ANSES Dozulé, data not

shown). Consequently, these methods are now approved by ANSES laboratory for diagnosis of *T. equigenitalis*.

Rapid detection of *K. pneumoniae* and *P. aeruginosa* within 1-2 hours by rtPCR (compared to 48h by culture) would improve pre-breeding screening for these pathogens and prevent unjustified use of antibiotics.

Unlike culture, the rtPCR method reported here provided a robust method to differentiate the two *Taylorella* species, with important implications for international trade. When compared to other assays, the increased sensitivity of this rtPCR might result in a higher number of positive results. Due to the grave implications to both the equine industry and international trade [2], all positive *T. equigenitalis* samples warrant further clinical investigation, molecular characterization and treatment [24].

5. Conclusion

An easy handling sample preparation (direct lysis) associated with two triplex rtPCR are proposed for rapid detection of *T. equigenitalis* and other organisms associated with infertility in mares. The duration of the assay is significantly reduced and the diagnostic specificity and sensibility are substantially increased when compared with the conventional column extraction method. This combination offers a good alternative to culture for pre-breeding screening to improve biosecurity practices on stud farms and for the international movement of horses. Multiplex assays for infectious diseases may become the principal screening methods in veterinary diagnostic laboratories.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Statement

None

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Author's contributions

AL, PG and BB participated to the conceptualization, methodology, supervision and project administration. LD, SC and YV performed formal analysis, data curation and validation. AL and BB coordinated the contributions from all co-authors. AL, PG and BB analyzed the data, discussed the results and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Ferris R, McCue P, Borlee G, Loncar K, Hennet M, Borlee B. *In vitro* efficacy of nonantibiotic treatments on biofilm disruption of gram-negative pathogens and an *in vivo* model of infectious endometritis utilizing isolates from the equine uterus. J Clin Microbiol. 2016;54:631-639.

2. Timoney P. Contagious equine metritis. Comp Immunol Microbiol Infect Dis 1996;19:199–204.

3. Delerue M, Breuil MF, Duquesne F, Bayon-Auboyer MH, Amenna-Bernard N, Petry S. Acute endometritis due to *Taylorella equigenitalis* transmission by insemination of cryopreserved stallion semen. J Equine Vet Sci 2019;78:10-13.

4. Jang S, Donahue J, Arata A, Goris J, Hansen L, Earley D, Vandamme P, Timoney P, Hirsh D. *Taylorella asinigenitalis* sp. nov., a bacterium isolated from the genital tract of male donkeys (Equus asinus). Int J Syst Evol Microbiol 2001;51:971–976.

5. Duquesne F, Hébert L, Breuil MF, Matsuda M, Laugier C, Petry S. Development of a single multi-locus sequence typing scheme for *Taylorella equigenitalis* and *Taylorella asinigenitalis*. Vet Microbiol 2013;167:609–618.

6. Arata A, Cooke C, Jang S, Hirsh D. Multiplex polymerase chain reaction for distinguishing *Taylorella equigenitalis* from *Taylorella equigenitalis* -like organisms. J Vet Diagn Invest. 2001;13:263-265.

7. Meade B, Timoney P, Donahue J, Branscum A, Ford R, Rowe R. Initial occurrence of *Taylorella asinigenitalis* and its detection in nurse mares, a stallion and donkeys in Kentucky. Prev Vet Med. 2010;95:292-296.

8. Moore J, Buckley T, Millar B. Molecular surveillance of the incidence of *Taylorella equigenitalis* and *Pseudomonas aeruginosa* from horses in Ireland by sequence-specific PCR. Equine Vet J. 2001;33:319-322.

9. Samper J, Tibary A. Disease transmission in horses. Theriogenology 2006;66: 551–559.

10. Allen J, Begg A, Browning G. Outbreak of equine endometritis caused by a genotypically identical strain of *Pseudomonas aeruginosa*. J Vet Diagn Invest 2011;23:1236–1239.

11. Ousey J, Palmer L, Cash R, Grimes K, Fletcher A, Barrelet A, Foote A, Manning F, Ricketts S. 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 2009;41:878–882.

12. Luddy S, Kutzler M. Contagious Equine Metritis Within the United States: A Review of the 2008 Outbreak. J Equine Vet Sci 2010;30:393–400.

13. Kinoshita Y, Niwa H, Katayama Y, Hariu K. Development of loop-mediated isothermal amplification methods for detecting *Taylorella equigenitalis* and *Taylorella asinigenitalis*. J Equine Vet Sci 2015;26:25–29.

14. Wakeley P, Errington J, Hannon S, Roest H, Carson T, Hunt B, Sawyer J, Heath P. Development of a real time PCR for the detection of *Taylorella equigenitalis* directly from genital swabs and discrimination from *Taylorella asinigenitalis*. Vet Microbiol 2006;118:247–254.

15. Nadin-Davis S, Knowles MK, Burke T, Böse R, Devenish J. Comparison of culture versus quantitative real-time polymerase chain reaction for the detection of *Taylorella equigenitalis* in field samples from naturally infected horses in Canada and Germany. Can J Vet Res. 2015;79:161-169.

16. May C, Guthrie A, Keys B, Joone C, Monyai M, Schulman ML. Polymerase chain reaction-based national surveillance programme to determine the distribution and prevalence of *Taylorella equigenitalis* in South African horses. Equine Vet J 2016;48:307-311.

17. Mawhinney I, Errington J, Stamper N, Torrens N, Engelsma MY, Roest HI. Pooling of genital swabs for detection by PCR of *Taylorella equigenitalis*, the cause of contagious equine metritis. Equine Vet J 2019;51:227-230.

18. OIE. Chapter 2.5.2: Contagious Equine Metritis. OIE Terrestrial Manual. 2012.

19. AFNOR NF U47-108: Requirements and recommendations for the isolate and identification of *Taylorella equigenitalis* from genital samples of Equidae.

20. Hébert L, Moumen B, Pons N, Duquesne F, Breuil MF, Goux D, Batto JM, Laugier C, Renault P, Petry S. Genomic characterization of the *Taylorella* genus. PLoS ONE 2012;7:e29953.

21. Cattoir V, Gilibert A, Le Glaunec JM, Launay N, Bait-Mérabet L, Legrand P. Rapid detection of *Pseudomonas aeruginosa* from positive blood cultures by quantitative PCR. Ann Clin Microbiol Antimicrob 2010;9:21.

22. Thong K, Lai M, Teh C., Chua K. Simultaneous detection of methicillin-resistant *Staphylococcus aureus*, *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* by multiplex PCR. Trop Biomed 2011;28:21–31.

23. AFNOR NF U47-600-2 norm: Requirements and recommendations for the implementation, the development and the validation of veterinary PCR for animal health analysis methods.

24. Duquesne F, Merlin A, Pérez-Cobo I, Sedlák K, Melzer F, Overesch G, Fretin D, Iwaniak W, Breuil MF, Wernery U, Hicks J, Agüero-García M, Frías-Serrano N, San Miguel-Ibáñez E, Patrasová E, Waldvogel AS, Szulowski K, Joseph M, Jeeba J, Shanty J, Varghese P, Hans A, Petry S. Overview of spatio-temporal distribution inferred by multi-locus sequence typing of *Taylorella equigenitalis* isolated worldwide from 1977 to 2018 in equidae. Vet Microbiol 2020;242:108597.

Supplementary material

Table S1: Results of analytical specificity of ADIAVET CEMO *Taylorella* REAL TIME and ADIAVET KLEB/PSEUDO REAL TIME PCR.

1 **Table 1.** Characteristics of PCR and the methods according to the pathogen

		T. equigenitalis		*T. asinigenitalis*		*K. pneumoniae*		*P. aeruginosa*	
		Swab	**Semen**	**Swab**	**Semen**	**Swab**	**Semen**	**Swab**	**Semen**
A. Characterization of PCR									
Efficiency (%)		97		91		79		82.5	
R^2		0.99		0.99		0.99		0.99	
Repeatability (range of CV %)		0.9-1.7		0.9-3.2		1.3-2.9		1.2-3.4	
Reproducibility (range of CV %)		2-3.1		3.3-4.5		0.9-1.4		2.8-3.6	
Analytical specificity		OK		OK		OK		OK	
LOD_{PCR} (GE/reaction)		5		20		20		20	
B. Characterization of methods									
LOD_{Method} (CFU/ml)	Direct lysis	750	/	2750	/	100	/	7500	/

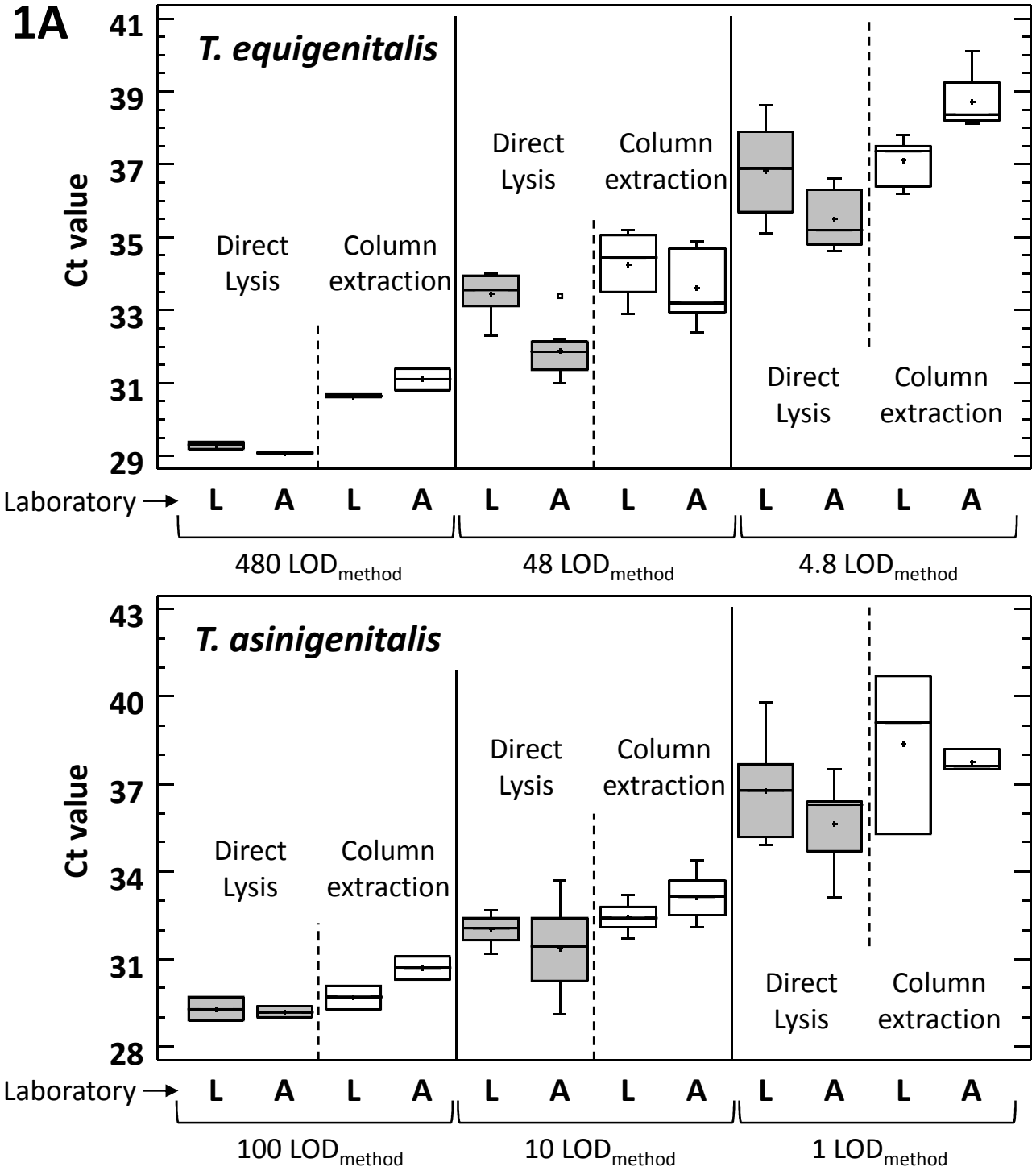
		Column purification	750 3000	2750 550	500 5000	7500 15000
DSe (%)		Direct lysis	ND	ND	100 [84.5;100]	100 [72.2;100]
		Column purification	ND	ND	100 [84.5;100]	100 [72.3;100]
DSp (%)		Direct lysis	100 [99.2;100]	100 [99.2;100]	92.7 [90;94.7]	99 [97.6;99.6]
		Column purification	100 [99.2;100]	100 [99.2;100]	93.1 [90.5;95.1]	99.4 [98.2;99.8]

LOD_{PCR}: Limit of detection PCR; GE: genome equivalent; LOD_{Method}: Limit of detection method; Dse: Diagnostic sensitivity; Dsp: Diagnostic specificity; ND: Not Determined; /: Not realized; CV: Coefficient of variation; CFU: Colonies Forming Units; []: confidence interval

Figure legend

Figure 1. Results of the inter-laboratory comparison assay (1A). Box and whiskers plot presentation of Ct values obtained for *T. equigenitalis* (top graphic) and *T. asinigenitalis* (bottom graphic) using direct lysis (grey plots) or column extraction (white plots). The rectangle represents 50% of observed responses for each group, horizontal line indicates median of the group, and the cross indicates the average of the group. Square indicates outlier results. L = LABÉO, A = ADIAGENE. Multifactorial ANOVA of Ct values obtained for *T. equigenitalis* and *T. asinigenitalis* (1B), with spiking levels, Laboratories and purification methods (direct lysis or column purifications) used as factors. Statistically significant differences are indicated in bold text. The contribution of each factor was measured having removed the effects of all other factors

1A



1B

		T. equigenitalis		*T. asinigenitalis*	
Factors	Details	Ct (average)	p-value	Ct (average)	p-value
Spiking levels	early	30.0±0.35	**<0.0001**	29.7±0.43	**<0.0001**
	intermediate	33.3±0.18		32.2±0.22	
	late	36.9±0.2		37.0±0.26	
Laboratories	LABEO	33.7±0.19	**0.0173**	33.2±0.24	0.2921
	Adiagène	33.1±0.2		32.8±0.24	
Purification methods	Direct lysis	32.7±0.19	**<0.0001**	32.3±0.22	**0.0001**
	Column	34.1±0.2		33.6±0.26	