VALIDATION OF NEW MULTIPLEX qPCR TECHNIQUES FOR THE DIAGNOSIS OF ABORTIVE DISEASES

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Introduction

Among ruminants, abortion is a pathological event that affects productivity and causes considerable direct and indirect economic losses to farmers because of foetal death and the diagnostic, therapeutic and prophylactic costs. For these reasons, an accurate and fast diagnosis is always desirable to establish effective control measures. Microbial agents, such as *Chlamydophila spp.*, *Coxiella burnetii, Brucella abortus, Campylobacter fetus*, *Leptospira spp.*, *Listeria monocytogenes, Salmonella spp.* or parasites such as *Neospora caninum*, *Toxoplasma gondii*, *Anaplasma phagocytophilum,* are among the main infectious causes of abortion and require rapid and reliable diagnosis.
About 50% of the causes of abortions are not due to an infectious agent. In 10-15% of abortions the etiological agent can be identified, while 35-40% of cases of abortion remain unsolved.

Diagnostic tools are essential for **the detection and the monitoring of abortive pathogens**, and can play an important role to prevent the spread of the diseases. IDvet has developed an abortive pack consisting of 7 real-time PCR diagnostic tools.

- ID Gene ® Anaplasma phagocytophilum Duplex (IDANA)
- ID Gene® Chlamydophila spp Duplex (IDCHLM)
- ID Gene ® Q Fever Triplex (IDQF)
- ID Gene ® Q Fever-Chlamydophila spp Triplex (IDQFCH)
- ID Gene ® Neospora caninum Duplex (IDNEO)
- ID Gene ® Toxoplasma gondii Duplex (IDTOXO)
- ID Gene ® Brucella spp Triplex (IDBRU)

Abortive pack by Real-Time PCR

The kits from the **abortive pack are ready-to-use** qPCR kit assays. These kits can be used to test **ruminant whole blood**, **swabs** (endo-cervical, vaginal) and **organs**. They simultaneously amplify target DNA and an endogenous control to **validate all analytical steps of the system**. In addition, IDQF and IDBRU also target an exogenous internal control. Results may be obtained in **less than 2 hours :** extraction in 20 minutes (fully automatable with the **IDEAL™** extraction robot), and amplification in 60 minutes.

MAGFAST™ universal for automated DNA/RNA extraction

qPCR kit with calibrated sentinel/positive control

The IDEAL™ extraction robot

Rigorous system of controls:
- ▶ Endogenous internal control that confirms the presence of cells and the quality of the sample.
- ▶ Exogenous internal control to validate both extraction and amplification steps.
- ▶ Freeze-dried calibrated positive control supplied in the kit : a sentinel to check run to run reproducibility over time.

Validation of both kits

▶ Analytical Specificity

The specificity of the 6 qPCR systems was evaluated in silico by aligning the target PCR systems (primers and probes) with the databases available on the NCBI (National Center for Biotechnology Information). After alignment, 100% in silico specificity was found for each specific target. Alignments do not show high sequence homology with pathogens from the same ecological niche.

▶ Inclusivity

Inclusivity was evaluated on a panel of 3 *Coxiella burnetii* strains (Nine mile strain), 21 *Chlamydophila spp* strains, 1 *Neospora caninum* strain, 2 *Toxoplasma gondii* strains, 2 *Anaplasma phagocytophilum* strains and 23 *Brucella spp strains*. This isolates have been provided by different reference laboratories.

▶ Exclusivity

The abortive pack was evaluated on a panel of DNA and RNA. Nucleic acid extraction was performed by magnetic beads (MAGFAST kit) as per manufacturer's instructions. Exclusivity was assessed on a panel of about 50 pathogens, close to each pathogens of interest or present in the same ecological niche.

▶ The abortive pack identified all abortive pathogens of interest tested and did not show any cross-reactions with the other pathogens tested.

▶ PCR characteristics

- The limit of detection of the PCR (LD_{PCR}) is the smallest number of copies of target nucleic acid per unit volume that can be detected in 95% of cases.
- To determine the method detection limit (**MLD**) on MAGFAST, serial dilutions of quantified bacterial strains are prepared to spike negative matrix at different concentration levels.
- For robustness, variations of temperature of +/- 1° C for each PCR step, and variations of nucleic acid volume (4.5µl, 5µl and 5.5µl) do not affect the kits. Results in table below.

Performances	IDANA	IDCHLM	IDQF	IDNEO	IDTOXO	IDBRU
Analytical sensitivity (LD_{PCR})	5 copies/PCR	2,5 copies/PCR	2,5 GE/PCR	2,5 copies/PCR	5 copies/PCR	12,5 copies/PCR
Efficiency	101,1 %	103,4 %	99,2 %	101,6 %	102,6 %	103,1 %
Method detection (MDL) with MAGFAST	500 copies/ml	500 copies/ml	500 GE/ml	2250 copies/ml	600 copies/ml	7000 CFU/ml
Robustness	Unaffected by all parameters tested CV% < 5%					

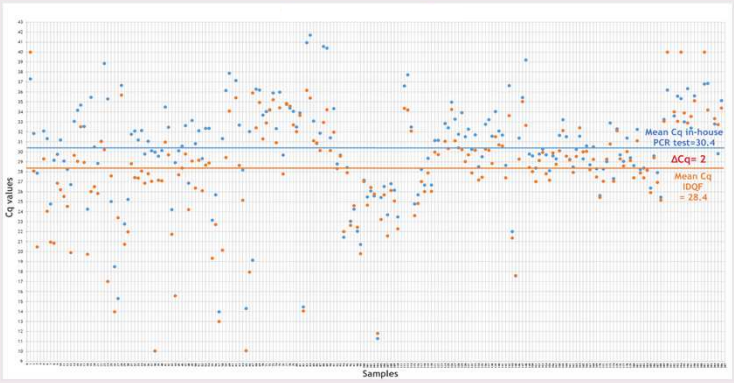
▶ The LD_{PCR} of those assays were between 2,5 to 12,5 copies/PCR.
▶ The MAGFAST MLD is less than or equal to 2250 copies/mL.

▶ Diagnostic sensitivity

The status of the different sample types were previously characterized by other methods
Results in table below :

	Number of samples tested	Specificity	Sensitivity
IDANA	52 blood and swab samples	100 %	100 %
IDCHLM	40 swab and 43 organ and tissue samples	100 %	100 %
IDQF	13 organ, 13 milk, 16 faeces and 292 swab samples	100 %	100 %
IDNEO	75 organ and tissue, 9 swab and stomacal liquid samples	97 % [91,1;100]	100 %
IDTOXO	46 organ and tissue, 8 swab and stomacal liquid samples	95 % [84,7;100]	100 %
IDBRU	260 milk samples	96 % [92,2;99,8]	83 % [51,6;97,9]

Exemple of results for IDQF below :



Comparison of results obtained for 207 vaginal swab samples from sheep, goat and cattle using the Panning et al., 2008 PCR and the IDvet IDQF PCR kit.

Conclusion

All PCR kits from abortive pack demonstrate excellent specificity. These kits successfully detect the 6 pathogens of interest (*Coxiella burnetii*, *Anaplasma phagocytophilum*, *Chlamydophila spp*, *Neospora caninum*, *Toxoplasma gondii* and *Brucella spp)* and show equivalent sensitivity compared to reference/competitors PCR methods tested. The abortive pack is an essential tool for monitoring and management of abortions in herds.

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