DEVELOPMENT AND VALIDATION OF A NEW TUBERCULOSIS REAL TIME PCR KIT

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ABSTRACT

Bovine tuberculosis (TB) is a chronic disease of animals caused primarily by *Mycobacterium bovis*. The disease causes general state of illness, coughing and eventually death. It can affect cattle, but also wild animals and is an important issue threatening public health. In this context, monitoring of these bacteria calls for diagnosis with reliable, sensitive and specific tools.

Applied Biosystems™ VetMAX™ M. tuberculosis Complex kit (MTBC), a new molecular tool, allows the simultaneous detection of Mycobacteria belonging to *Mycobacterium tuberculosis* complex (MTBC) and an internal control. To demonstrate the kit's performances, verification studies were carried out on cattle and wild animals, such as cervids, wild boars and badgers.
41 strains including 22 non MTBC *Mycobacteria* were tested to evaluate the specificity of the assay. More than 750 negatives and 350 positives field samples (lymph nodes and surrounding tissue) were tested in collaboration with partner labs to evaluate diagnostic sensitivity and specificity in respect to their country requirements.

Results obtained from the tested strains showed that the MTBC kit is specific for *Mycobacterium* complex and does not detect other tested pathogens. Results obtained on field samples showed a correlation of more than 95% with other methods tested.

In the context of increasing of TB prevalence, detection and confirmation of TB infection need diagnostic tools that are easy to handle by labs, which provide sensitive, reliable and fast results in order to help ensure the efficacy of surveillance and control programs. The qPCR kit VetMAX M. tuberculosis Complex kit, was designed to meet these expectations.

INTRODUCTION

Bovine tuberculosis (TB) is a chronic disease of animals caused primarily by *Mycobacterium bovis*. The disease causes general state of illness, coughing and eventually death. It can affect cattle, but also wild animals and is an important issue threatening public health. In this context, monitoring of these bacteria calls for diagnosis with reliable, sensitive and specific tools.

MATERIALS AND METHODS

VetMAX M. tuberculosis Complex kit is a TaqMan® ready-to-use real-time PCR assay based on the simultaneous detection of Mycobacteria belonging to *Mycobacterium tuberculosis* Complex (MTBC) and an exogenous Internal Positive Control (IPC).
For the development of a reliable, sensitive and specific rtPCR system, a large panel of Mycobacteria belonging or not to the MTBC, and other closed pathogens were tested. This product fulfills all the validation criteria of PCR characteristics and complete method, as required by the NF U47-600-2 standard.
The isolation of DNA from field samples carried out on bovine and wild animals, such as cervids, wild boars and badgers, was performed with MagVet™ Universal isolation kit. More than 750 negative samples and 350 positive samples (lymph nodes and surrounding tissues) were collected by different partners and tested to evaluate diagnostic specificity and sensitivity, in respect to their country requirements.

Figure 1. M. tuberculosis diagnostic tools

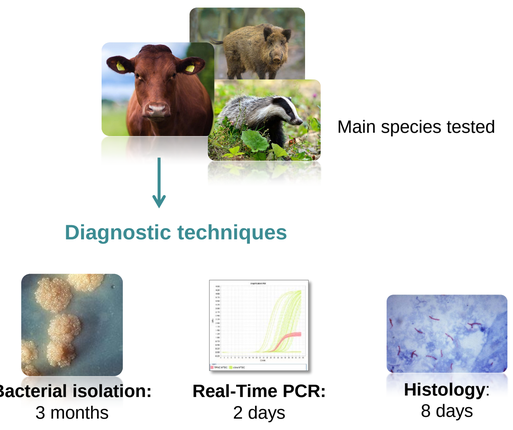
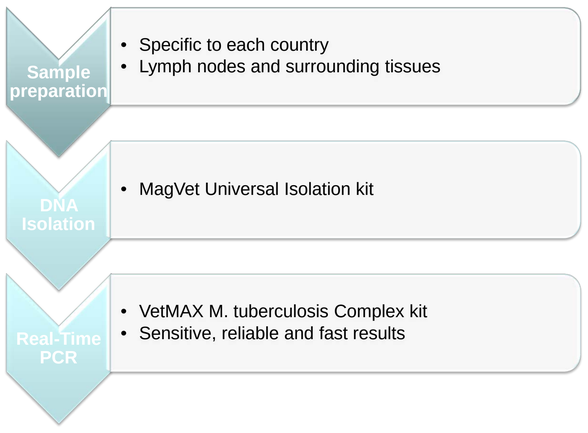


Figure 2. PCR Complete workflow



RESULTS

Table 1. Analytical Specificity of VetMAX M. tuberculosis Complex kit (MTBC)

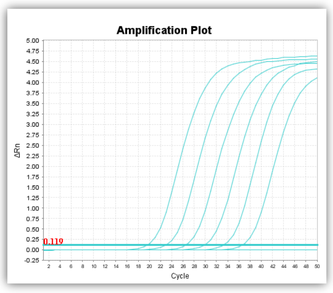
	Strain	MTBC detection
Inclusivity	*Mycobacterium bovis*	Detected
	Mycobacterium tuberculosis	
	Mycobacterium africanum	
	Mycobacterium microti	
	Mycobacterium caprae	
	Mycobacterium pinnipedii	
Exclusivity	22 Mycobacteria not belonging to MTBC (eg: xenopi)	Not detected
	19 other pathogens	

The inclusivity of VetMAX M. tuberculosis Complex kit is evaluated on Mycobacterium belonging to the M. tuberculosis complex coming from different commercial supplier (Pasteur; DSMZ). As indicated in the table above, the kit detect all MTBC Mycobacteria tested.

The exclusivity is assessed on a panel of 22 Mycobacteria not belonging to the MTBC and 19 other pathogens close to M. tuberculosis complex, either because they are preferentially found in the same ecological niches, phylogenetically close, or because they have the same clinical symptoms in target species. None of the strains tested is detected.

VetMAX M. tuberculosis Complex kit is specific for M. tuberculosis complex and does not detect other tested pathogens.

Figure 3. Efficiency of VetMAX M. tuberculosis Complex kit (MTBC)



R2=0.998; Eff=99.14%

The PCR efficiency of VetMAX M. tuberculosis Complex kit, assessed from serial dilutions of a quantified MTBC plasmid (pTUB) until signal of extinction, tested in triplicate, is close to 100 %.

Table 2. Characteristics of VetMAX M. tuberculosis Complex kit according to French Standard for Veterinary PCR (NF U47-600-2)

Characteristics	MTBC Validation
Analytical specificity (Table 1)	100 %
Efficiency (Figure 3)	Close to 100 %
Limit of detection	16 copies/PCR
Repeatability	CV < 1.55 %
Intermediate precision	CV < 1.52 %
Robustness	Unaffected by all parameters tested
Experimental LOD – Lymph nodes Magnetic beads purification	1 UG/µL of spiked sample
Experimental LOD – Lymph nodes Columns purification	2 UG/µL of spiked sample

The limit of detection (LOD), evaluated on a quantified MTBC plasmid, is estimated to be 16 copies per PCR.

Repeatability and intermediate precision are evaluated on quantified MTBC plasmid at different concentration level and all coefficients of variation obtained are less than 1.55 %.

For robustness, variations of temperature of hybridization (59° C, 60° C and 61° C), time of hybridization (54 sec, 60 sec and 66 sec), mix of volume (18µL, 20µL and 22µL) and nucleic acid volume (4.5µL, 5µL and 5.5µL) do not affect the MTBC PCR.

To determine the experimental limit of detection, serial dilutions of quantified plasmid are prepared to spike negative matrix (lymph nodes) at different concentration levels. The detection limit of MagVet Universal Isolation kit is determined at 1 UG/µL of spiked sample and 2 UG per microliter of spiked sample with QIAamp® DNA mini kit.

Table 3. First evaluation of VetMAX M. tuberculosis Complex kit (MTBC)

- **Samples collected**

Samples collected	
• Bovine	287
• Wild boar	106
• Badger	20
• Cervid	7

- **Results obtained**

		TUB2W		
		Detected	Not detected	Total
MTBC	Detected	31	1	32
	Not detected	3	385	388
	Total	34	386	420

Statistical equivalence (5 % risk of error - Chi2 test).
Kappa correlation coefficient = 0.93; meaning "almost perfect agreement" of these both methods for detection of MTBC complex on lymph nodes.

Table 4. External partner lab evaluation of VetMAX M. tuberculosis Complex kit (MTBC)

- **Samples collected**

H2SO4/NaOH sample preparation	
• Bovine	445
• Wild boar	2
• Badger	82

PBS1X sample preparation	
• Bovine	148
• Wild boar	2
• Badger	3

- **Results obtained**

H2SO4/NaOH sample preparation		TUB2W		
		Detected	Not detected	Total
MTBC	Detected	164	6	170
	Not detected	8	351	359
	Total	172	357	529

Statistical equivalence (5 % risk of error - Chi2 test).
Kappa correlation coefficient = 0.94; meaning "almost perfect agreement" of these both methods for detection of MTBC complex on lymph nodes.

Diagnostic sensitivity = 95.4 % [91.0-98.0 %]
Diagnostic specificity = 98.3 % [96.4-99..4 %]

PBS1X Sample preparation		TUB2W		
		Detected	Not detected	Total
MTBC	Detected	143	0	146
	Not detected	0	10	10
	Total	143	10	153

Statistical equivalence, (5 % risk of error - Chi2 test.
Kappa correlation coefficient = 1.0; meaning "very good" correlation of these both methods for detection of MTBC complex on lympnodes.

Diagnostic sensitivity = 100.0 % [97.5-100.0 %]

CONCLUSIONS

VetMAX M. tuberculosis Complex kit is real-time PCR kit allowing the simultaneous detection of Mycobacterium tuberculosis complex (MTBC) and an exogenous positive control in lymph nodes and surrounding tissues samples.

The kits fulfills all the validation criteria for PCR characteristics and complete method required by the French standard (NF U47-600-2) « Requirements and recommendations for the development and validation of qRT-PCR in Animal Health ».

The specificity evaluated on different strains showed no cross-reactions with closely related pathogens or Mycobacteria not belonging to the MTBC. This kit had an efficiency close to 100 % and its PCR limit of detection is 16 copies per PCR (95% confidence interval). The experimental LOD is 1 or 2 UG per microliter of spiked matrix according to purification test used. Tests results on field samples showed an "almost perfect agreement" with other method tested in each study. The diagnostic sensitivity was evaluated at 97.5 % [95.1-98.9 %] and diagnostic specificity at 98.4 % [96.5-99..4 %].

In the context of increasing of TB prevalence, detection and confirmation of TB infection in a suspicious animals need diagnostic tools that are easy to handle by labs, which provide sensitive, reliable and fast results in order to help ensure the efficacy of surveillance and control programs. The qPCR kit VetMAX M. tuberculosis Complex kit, was designed to meet these expectations and completes the tuberculosis solution portfolio for Thermo Fisher Scientific.

REFERENCES

- NF U47-600-2 – Animal Health analysis methods – PCR Part 2: Requirements and recommendations for the development and validation of veterinary PCR (http://www.afnor.org)

ACKNOWLEDGEMENTS

- French Veterinary Lab – Dordogne (24)
- French Veterinary Lab – Côte-d'Or (21)
- French Veterinary Lab – Pyrénées-Atlantiques (64)

TRADEMARKS/LICENSING

- Applied Biosystems™ VetMAX™ M. tuberculosis Complex kit (Cat. No. MTBC)*
- MagVet™ Universal Isolation kit (Cat. No. MV384)

* For Veterinary use only. For *in vitro* use only.

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