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Shaken, not stirred: magnetic bead DNA extraction as a rapid and effective method for the scaling up of bovine tuberculosis diagnosis

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Abstract

Background The growing use of real-time PCR (qPCR) as a diagnostic method for bovine TB (bTB) requires rapid and effective DNA extraction methods, which are crucial for its success. Automated DNA extraction methods based on magnetic beads are a promising alternative to conventional silica column-based protocols (COL protocol) due to their high throughput capacity and reduced hands-on time. This study aimed to assess the performance of the MagMax CORE Nucleic Acid Purification kit and the KingFisher Flex instrument (KF protocol) as an alternative for scaling up the use of qPCR in bTB diagnosis.

Methodology Performance was evaluated with two different real-time PCR (qPCR) protocols, based on the IS*6110* element and the QuantiFast and VetMAX™ (QF and VM protocols) kits, on 145 frozen tissue homogenates confirmed as either bTB-positive or negative through a composite reference standard based on microbiological culture, column-based extraction, and qPCR, as well as on negative tissue samples spiked with 10^6 to 10^3 CFU/ml of *M. bovis* BCG.

Results The performance of both qPCR protocols was very high on samples extracted using the KF protocol, with positive percent agreement (PPA) values of 89.04% [95% Confidence Interval (CI): 79.54–95.15%] and 93.15% [95% CI: 84.74–97.74%] for the QF and VM protocols, respectively, and negative percent agreement (NPA) values of 100% [95% CI: 95.01–100.00%]. A higher variability was identified in samples analysed with the same qPCR protocol but different extraction methods. Higher Ct values were identified for samples extracted using the KF protocol in both routine and spiked samples, likely due to using the same amount of starting material for both extraction methods, which was lower than recommended by the manufacturer for the KF protocol.

Discussion The results of this study indicate that the MagMAX CORE Nucleic Acid Purification kit coupled with a KingFisher Flex instrument is a valuable alternative for the extraction of MTBC DNA from bovine tissues. However, the increased variability and Ct values suggest that a larger amount of starting material is recommended for this methodology, warranting further studies.

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Keywords Real-time PCR, Magnetic bead extraction, Bovine tuberculosis, Molecular detection, Mycobacterium tuberculosis complex, Mycobacterium bovis, Direct tissue extraction, Cattle

Background

Eradication of tuberculosis (TB) in domestic cattle has been a central goal of veterinary institutions across the globe for decades [1–4], not only due to its impact in animal health and productivity, which is essential in economies that depend on livestock, but also due to its clear implications in public health as a zoonosis. These efforts have been recently recognised by the WHO's Roadmap for Zoonotic TB [5], in which the reduction of TB prevalence in livestock has been considered as one of the 10 priorities for tackling zoonotic TB.

In high-income settings, bovine TB (bTB) eradication programmes are usually based on a test-and-slaughter strategy in which infected animals are detected using official *ante mortem* techniques, such as the intradermal tuberculin test. In the European Union (EU), significant improvements in the eradication, control and surveillance of TB in cattle have been implemented through the application of Regulation (EU) 2016/429 *on transmissible animal diseases* [6]. Currently, the detection of *Mycobacterium tuberculosis* complex (MTBC)-specific nucleic acids in animal tissues through qPCR has been included for the confirmation of suspected cases and the withdrawal of the Official Tuberculosis Free status, due to its reduced turnaround times, flexibility and, at least, similar sensitivity and specificity to bacteriological culture [7–9]. In addition to the plethora of in house methods available in scientific literature [7, 8, 10–12], several companies have released ready-to-use reagents for the detection of MTBC members in animal tissues, such as the VetMax™ *M. tuberculosis* Complex kit [11, 13] or the ID Gene™ *Mycobacterium tuberculosis* complex Duplex kit (Innovative Diagnostics).

The direct detection of pathogens requires the extraction of nucleic acids from biological samples, and the protocols employed for this purpose have a critical effect on the yield and quality of the resulting material [14]. Currently, the manual purification of DNA/RNA through silica columns is the most extended extraction method [14], usually involving preliminary mechanical and/or biochemical lysis steps for the detection of intracellular and robust pathogens, such as *Mycobacterium bovis* [15]. Although these methods are easy to perform and cost-effective, their yield depends on the elution volume and column integrity, which can be affected by the amount of processed sample, the type of sample matrix or the reagents used, among others. In addition, the manual nature of these methods makes their use in high throughput settings a more cumbersome approach.

Magnetic bead-based extraction methods have become a popular alternative for the separation and purification of nucleic acids in complex mixtures, especially for viral pathogens [16–18], due to their reduced costs and tuneable chemistries [19]. These methods are based on the use of positively-charged magnetic beads which bind to DNA and are anchored to a magnet during the washing and purification steps. An important advantage of these protocols is that many commercial kits couple their use with automatized robotic platforms, therefore reducing the processing time and allowing for a higher sample throughput, since many perform the extraction on multi-welled plates. The utility of these approaches in the diagnosis of infectious diseases has been exemplified during the Covid-19 pandemic, in which such methods allowed the adaptation of laboratorial testing capacity as the epidemiological situations changed with time [20].

The increasing relevance of qPCR in the diagnosis of TB in animals will require the implementation of rapid and effective methods of DNA extraction. Automated magnetic bead-based methods may be an interesting approach for the rapid and scalable extraction of MTBC-specific nucleic acids. An example of such methods are the KingFisher instruments (Flex, mL or Duo Prime), which coupled with the MagMAX CORE Nucleic Acid Purification kit, allow for the automatic purification of up to 96 tissue homogenates obtained from 2 to 5 g of animal tissues (lymph nodes and surrounding tissues) in approximately 20 min.

The objective of this study was to evaluate the performance of the VetMax™ *M. tuberculosis* complex kit and the qPCR protocol recommended by the European Union Reference Laboratory (EU-RL) for bTB on DNA extracted using the MagMax CORE Nucleic Acid Purification kit on a KingFisher Flex instrument (ThermoFisher Scientific), as well as on DNA extracted using the DNEasy Blood & Tissue kit (Qiagen).

Results

Performance of the column-based protocol on bovine tissue samples

A total of 145 samples were randomly selected from the routine diagnostic workflow for bTB at VISAVET and extracted using protocol COL, of which 71 were positive to the QF qPCR and 74 were negative. A total of 66 QF qPCR-positive samples were confirmed through microbiological culture, whereas five were confirmed through the IS*1081* qPCR (Table 1). In addition, two QF qPCR-negative samples were positive for culture. As a result, the PPA and NPA values between qPCR and culture

Table 1 Agreement between the QF qPCR (IS*6110*) and microbiological culture on samples extracted using the column-based approach (protocol COL)

		QF qPCR (IS*6110*)			Positive Percent Agreement (95% CI)	Negative Percent Agreement (95% CI)	Kappa (95% CI)
		Positive	Negative	Total			
Culture	Positive	66	2	68	92.96% (84.33–97.67%)	97.47% (91.15–99.69%)	0.903 (0.834–0.973)
	Negative	5*	72	77			
	Total	71	74	145			

**These samples were also positive to the IS1081 QF qPCR*

Table 2 Agreement between the VM qPCR and the true status of infection on samples extracted using the column-based approach

		True status			Positive Percent Agreement (95% CI)	Negative Percent Agreement (95% CI)	Kappa (95% CI)
		Positive	Negative	Total			
VM qPCR	Positive	70	1*	72	95.89% (88.46–99.14%)	98.61% (92.50–99.96%)	0.945 (0.892–0.998)
	Negative	3**	71	74			
	Total	73	72	145			

** This sample showed amplification for the QF qPCR with a Ct value above its diagnostic threshold (Ct > 38.0).*

*** Two samples were culture positive, whereas one was QF qPCR positive and IS1081 positive.*

Table 3 Agreement between the qPCR results and the true status of infection in samples extracted using the magnetic bead-based protocol

		True status			Positive Percent Agreement (95% CI)	Negative Percent Agreement (95% CI)	Kappa (95% CI)
		Positive	Negative	Total			
QF qPCR	Positive	65	0	65	89.04% (79.54–95.15%)	100% (95.01–100.00%)	0.895 (0.825–0.965)
	Negative	8	72	80			
	Total	73	72	145			
VM qPCR	Positive	68	0	68	93.15% (84.74–97.74%)	100.00% (95.01–100.00%)	0.931 (0.872–0.990)
	Negative	5	72	77			
	Total	73	72	145			

were 92.96% (95% Confidence Interval or CI: 84.33–97.67%) and 97.47% (95% CI: 91.15–99.69%), respectively. According to these analyses, a total of 73 samples were classified as true positive for this study, whereas 72 were classified as true negative.

When these samples were analysed with the VM qPCR kit, 70 true positive samples were positive whereas three were negative (Table 2). In addition, 71 true negative samples were also negative to the VM qPCR kit, whereas one was positive. As a result, the PPA and NPA values of the VM qPCR kit with respect to the true infection status of the samples were 95.89% (95% CI: 88.46–99.14%) and 98.61% (95% CI: 92.50 to 99.96%), respectively. When both qPCR kits were compared, agreement (κ) between methods was 0.945 (0.892 to 0.998) (Supplementary Table 1).

Performance of the magnetic bead-based protocol on bovine tissue samples

When the samples were extracted using protocol KF, 65 true positive samples were QF qPCR-positive, whereas 8 were QF qPCR-negative (Table 3). All true negative samples (n=72) were also QF qPCR-negative. Positive and NPA values were 89.04% (95% CI: 79.54–95.15%) and 100% (95% CI: 95.01–100.00%), respectively, whereas the total agreement was 0.895 (95% CI: 0825 to 0965). Six out of the eight discrepant results were confirmed by microbiological culture, whereas two had been positive to the QF qPCRs (IS*6110* and IS*1081*) when extracted using the COL protocol. In addition, one of the culture-negative samples had tested positive to the VM qPCR when extracted using the COL protocol.

When the DNA samples were tested using the VM qPCR, 68 true positive samples were positive, whereas five were negative (Table 3). Seventy-two true negative samples were also negative to the VM qPCR and no false-positives were detected. As a result, PPA and NPA values were 93.15% (95% CI: 84.74–97.74%) and 100.00% (95% CI: 95.01–100.00%), respectively, and total agreement was 0.931 (95% CI: 0.872 to 0.990). Three out of five discrepant results were confirmed by microbiological culture, whereas the remaining two had been positive to the QF IS*6110* and IS*1081* qPCRs (n = 2) or VM qPCR (n = 1), respectively, when extracted using the COL protocol.

Evaluation of Ct differences between extraction and qPCR protocols

Sixty-two of the 73 true positive samples were positive when extracted and analysed using both extraction (COL or KF) and qPCR (QF and VM) protocols. A pairwise sample comparison between the Ct values obtained for each combination of qPCR and extraction protocols revealed a high correlation between qPCR results obtained from samples extracted with the same extraction method (R^2=0.94 for the QF and VM qPCRs) (Fig. 1a), whereas correlation decreased for results obtained from the same qPCR protocol but different extraction method (R^2=0.64 and 0.59 for the QF and VM qPCRs, respectively). Although differences between the average Ct values obtained by either extraction and qPCR combination were not statistically significant (t-test; data not shown), there was a higher variability between Ct values obtained by either qPCR protocols when analysing samples from the COL and KF protocols, with a higher proportion of 1-2.99 and ≥3 cycle differences. This was also reflected in the higher limits of agreement in the Bland-Altman plots for the different extraction protocols, which ranged between −4.82 and 2.89 and −4.80 to 3.86 for the QF qPCR and VM qPCRs, respectively (Supplementary Fig. 1). In contrast, the limits of agreement ranged between −2.16 and 0.79 and −1.81 to 1.43 for extracts obtained using either the COL or KF protocols, respectively, but analysed using the QF and VM qPCR protocols.

Analysis of spiked tissue samples

For the COL protocol, the average Ct values for the QF and VM qPCR ranged between 25.64 (standard deviation or s.d. = 0.36) to 34.64 (s.d. = 0.77) and 26.37 (s.d. = 0.28) to 35.34 (s.d. = 0.79), respectively (Fig. 2). The average Ct values for the KF protocol ranged between 26.4 (s.d. = 0.06) and 37.07 (s.d. = 1.37) for the QF qPCR and 27.26 (s.d. = 0.41) and 38.04 (s.d. = 0.99) for the VM qPCR (Fig. 2). The mean Ct values for the QF and VM qPCRs were within 1.2 cycles of each other when analysing samples that were extracted with the same extraction protocol. When analysing samples from different extraction protocols, the mean Ct differences between the qPCR results varied with the bacterial load, being >1.5 cycles higher when extracted using the KF protocol at lower dilutions (10^4 and 10^3 CFU/ml).

Discussion

As a result of the implementation of Regulation (EU) 2016/429 and its delegated regulations, the availability of qPCR protocols will increase the use of these methodologies in veterinary laboratories throughout Europe in the coming years. Automated DNA extraction systems could be an interesting approach to answer this changing landscape, allowing for a high throughput of samples in a labour-effective manner. Although magnetic bead extraction was already described more than two decades ago for human TB [21], the use of this approach in bTB has been limited to date and focused on the use of sequence capture methods [22, 23]. This study described the evaluation of the KingFisher Flex instrument coupled with the MagMax CORE Nucleic Acid Purification kit as a possible alternative to the commonly used column-based methods, such as the Qiagen DNeasy Blood & Tissue kit [7, 24], one of the extraction protocols recommended by the EU-RL for bTB. This evaluation was performed using two different qPCR methods, both targeting the IS*6110* element, which have been validated for their use in the diagnosis of TB in bovines and other animal species [8, 11, 13, 25]. A total of 145 bovine tissue samples were selected from the routine diagnostic workflows of VISAVET during the bTB eradication campaigns of 2022–2023, reflecting the implementation of qPCR methods in a real-life setting.

Traditionally, the validation of molecular detection methods for the diagnosis of bTB has been performed using microbiological culture as the comparator test [7–10, 26, 27]. However, the change in legislation has led to the transition from culture-based methods to qPCR in Spain and other countries, reducing the use of this approach routinely. A composite reference standard approach was selected instead, which included the use of the IS*6110* qPCR (QF) and culture as diagnostic tests, and the IS*1081* qPCR as an additional test [25]. Overall, there was an excellent agreement between the QF qPCR and microbiological culture, with PPA and NPA values of 92.96% and 97.47%, respectively, in agreement with previous results [8]. Although agreement between culture and qPCR is remarkably high, there are certain factors that can negatively affect the performance of qPCR, such as the presence of PCR inhibitors, the absence of the genetic target or a decreased sensitivity in tissues with non-visible lesions as a result of the reduced amount of tissue that is processed during the DNA extraction in comparison to culture [7, 8, 28]. Thus, the combination of qPCR and microbiological culture could be an interesting approach under certain epidemiological scenarios [29].

The VM qPCR protocol also showed a very high performance, with PPA and NPA of 95.89% and 97.47%, respectively, when compared to the true status of the sample as defined by the composite reference standard. These results are in consonance with the remarkably high level of agreement (κ=0.945) between both qPCR methods, which could be related to the fact that they are based on the same mobile element (IS*6110*) and that both methods showed a similar limit of detection with 95% confidence (50 fg/µl of *M. bovis* BCG DNA or approximately 10 genomic equivalents) in an internal validation performed in VISAVET (data not shown). A large scale study

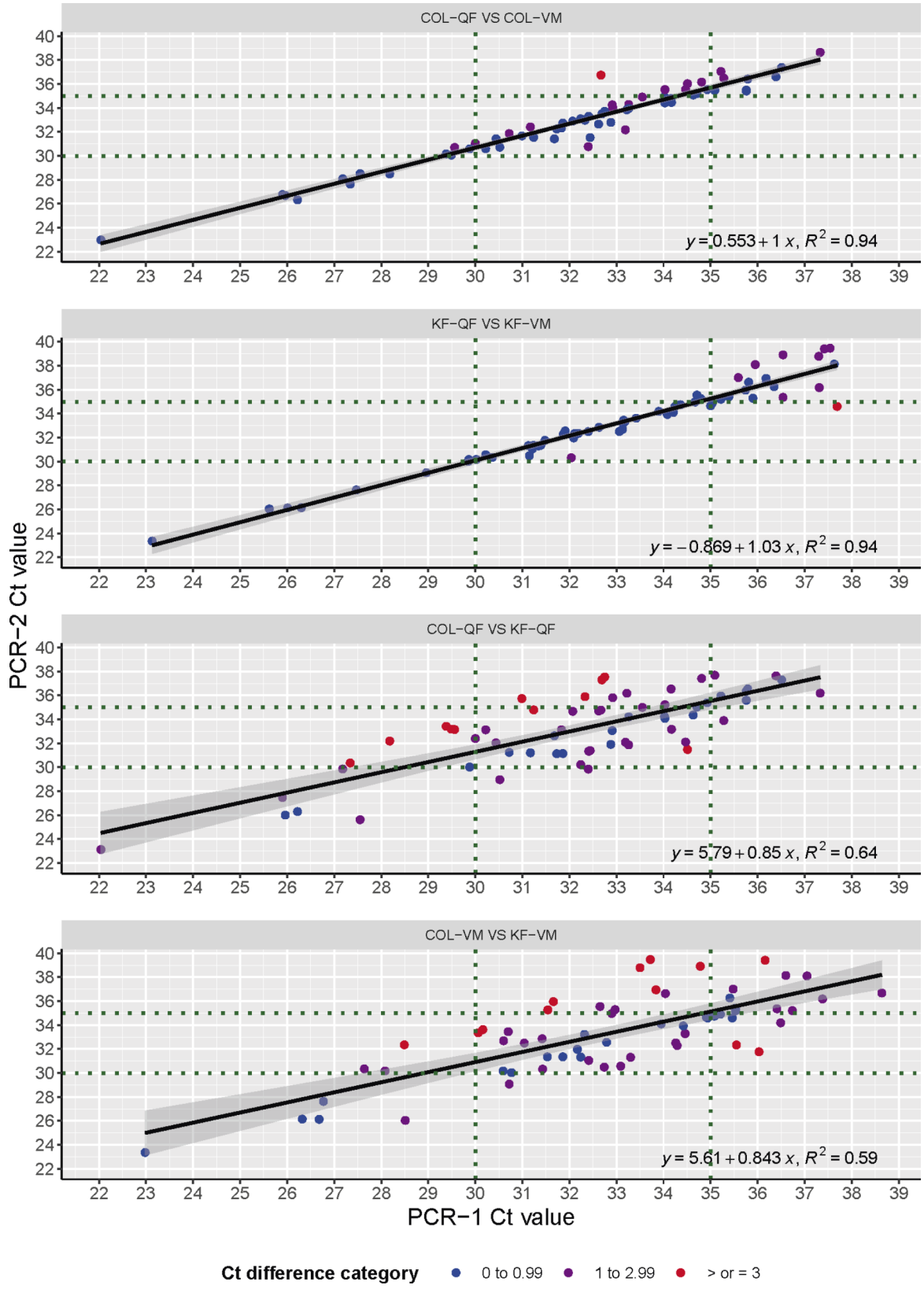

Fig. 1 Correlation analysis between sample pairs analysed by the different qPCR and DNA extraction protocols. Colours indicate the category of Ct difference between the elements of each pair. Grey lines indicate the 95% confidence interval. COL: Column protocol; KF: KingFisher protocol; QF: IS*6110* QuantiFast protocol (EU-RL); VM: VetMAX™*M. tuberculosis* Complex protocol

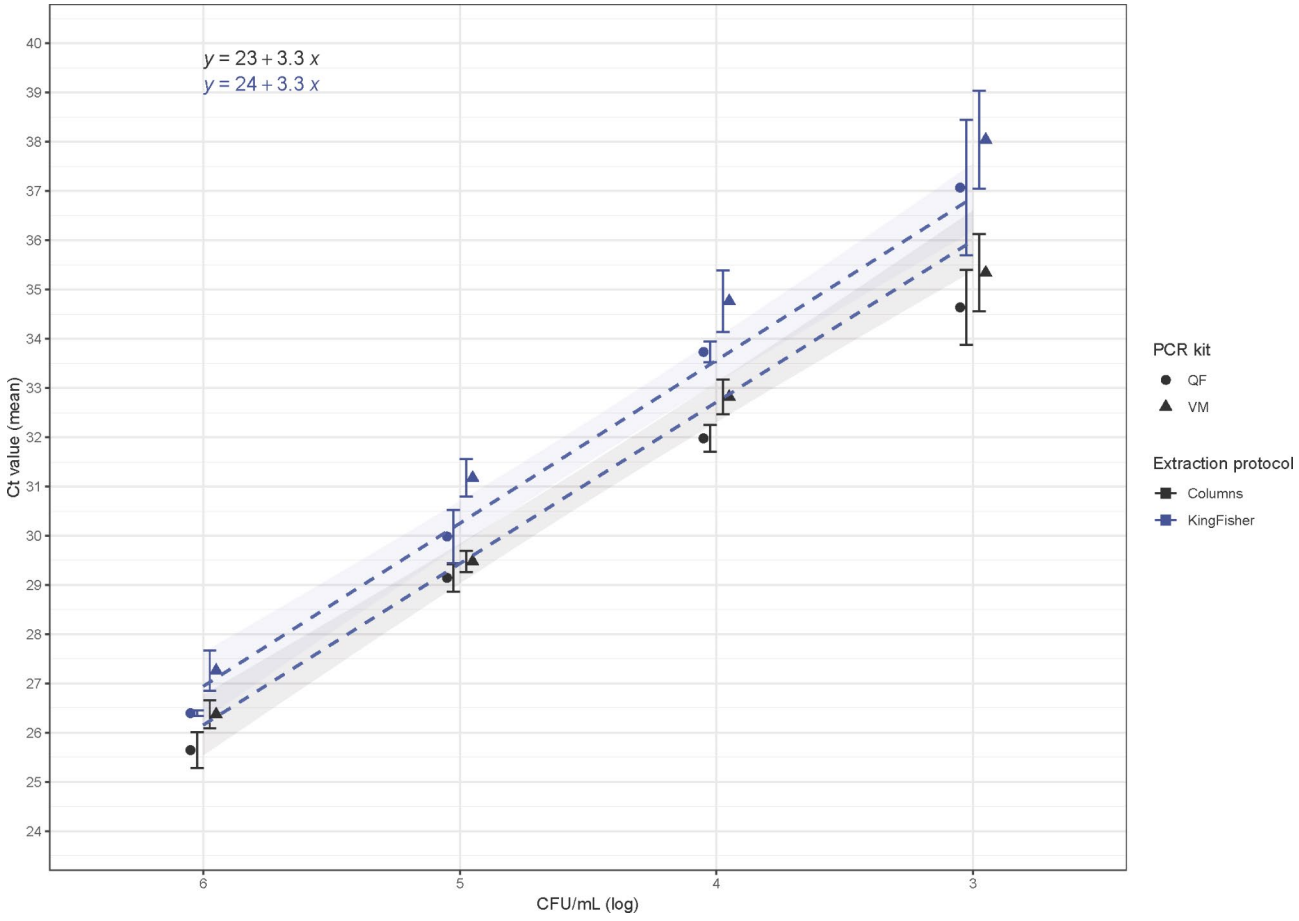

Fig. 2 Comparison of Ct values obtained for the QF and VM qPCR protocols on DNA extracted from a standard curve of tissue samples spiked with decreasing bacterial concentrations of *M. bovis* BCG ($10^6$to 10^3CFU/ml) using the COL or KF protocols

in France revealed a similar diagnostic specificity (97%) but a slightly lower diagnostic sensitivity (87.7%) [11], which could be related to several factors, including the different validation (Bayesian vs. reference-based) and sample selection approach, changes in reaction parameters since the release of the product or, more importantly, differences in the DNA extraction procedures employed in this study (QIAamp DNA mini kit and Magvet MV384 coupled with a King Fisher KF 96) and differences in the diagnostic cut-off (≤38 vs. ≤ 40; see below).

When the samples were extracted using the magnetic bead-based approach, the PPA values were also very high for the QF (89.04%) and VM qPCRs (93.15%), albeit slightly lower than the original results obtained with the column-based approach. The NPA value was 100%, with no false-negative results being identified by either method. Although the diagnostic performance was good for both approaches, the slight reduction in sensitivity prompted further research. A pairwise Ct comparison was performed on 62 true-positive samples that had been positive to both qPCR protocols using either extraction method, in order to assess any possible deviation in DNA yield or amplification efficiency. Although the bias between the different method combinations was within one cycle difference, which could be considered negligible and not affect the overall performance of the technique, a higher variability was identified for the qPCR values obtained with the KF protocol in comparison to the COL method.

In order to evaluate these differences in a controlled environment, both qPCR and DNA extraction methods were tested on a set of bovine tissue homogenates spiked with decreasing bacterial loads of *M. bovis* BCG (10^6 to 10^3 CFU/ml). Interestingly, the average Ct values for each qPCR were highly similar when analysing samples extracted using the same DNA extraction method. However, when comparing different extraction protocols, variability increased substantially, especially at lower bacterial loads (10^4 to 10^3 CFU/ml) and for the magnetic bead-based approach, in which average Ct values were up to 2.5 cycles higher than for the column-based approach. This could be in agreement with the increased proportion of sample pairs with ≥3 cycle differences (approximately 10-fold) in the field sample comparison. The fact that this

behaviour was similar for both qPCR methods suggests that differences could be related to a reduced DNA yield rather than to differences in qPCR chemistry or reaction conditions.

The apparent differences between the analytical properties of the magnetic bead protocol could be related to several factors associated to the experimental conditions that were used in this study or different parameters of the extraction method. Firstly, the DNA isolation methods optimized for the VM qPCR kit recommend the processing of 2–5 g of tissue, with no specific dilution factor. The column-based kit that was used for this comparison is based on the homogenization of 2 g of tissue in 12 ml of PBS, which is the starting point for both the microbiological culture and DNA isolation SOPs, defined as guidelines by the EU-RL for bTB. In order to avoid reprocessing the original tissue sample and introducing a much larger variability by testing a different portion, this homogenate was also used for the magnetic bead extraction. Even if the KF protocol recommends the use of a significantly higher amount of homogenate (1.8 ml) than the COL protocol (1 ml), the concentration may have been lower than the one recommended by the manufacturer, which could explain the differences in the analytical properties.

The average Ct value of the field samples was 32.8 (s.d. = 3.2), which indicates that they had, in general, relatively low bacillary loads. In addition, MTBC members are intracellular pathogens that, in certain cases, may be scarcely present in tissues [30, 31]. This entails that bacilli can be distributed irregularly in the tissue, even after homogenization, which could have led to additional variation when processing the sample. Thus, mechanical and biochemical lysis is crucial in order to guarantee the maximum yield of DNA for analysis. Although the COL and KF protocols used a mechanical and enzymatic lysis step, the intensity of the former is lower for the KF protocol (9,000 rpm vs. 6,800 rpm, respectively). Furthermore, an additional mechanical disruption step is recommended in the COL protocol after the overnight enzymatic digestion, which could additionally explain the difference in performance of both methods [15, 32].

One additional factor affecting qPCR performance could have been the introduction of PCR inhibitors due to differences in sample quantity or reagent composition [15, 33]. However, partial inhibitions (i.e., sample positive but IC negative or ≥33) were only detected for the QF qPCR, and approximately in 25% of the positive samples for either the column (n=17) and magnetic bead-based protocols (n=15), respectively (data not shown). Interestingly, only one sample pair with ≥3 cycle differences had shown any sign of inhibition, with the remaining inhibitory effects being distributed across the 0-0.99 and 1-1.99 dCt categories in a similar proportion (approximately 30%). No samples were inhibited for the VM qPCR for either extraction protocol, which indicates that this method could be less sensitive to inhibitors and that differences in the observed Ct values were probably not related to these phenomena.

The decreased analytical performance of the qPCR with samples extracted using the magnetic bead-based protocol in comparison to the ones extracted through the column-based approach did not affect the diagnostic performance results considerably, probably due to the fact that the cut-off for the VM qPCR recommended by the manufacturer was higher than the one recommended for the QF qPCR (≤40 vs. ≤ 38). Furthermore, Courcoul et al.., established a diagnostic cut-off of ≤38.0 in the large scale validation of the VM qPCR, which would have decreased sensitivity values to 82% if applied in this study (data not shown) [11]. When a ROC analysis was performed individually on the qPCR results obtained from the magnetic bead extractions, the diagnostic cut-off values were set to ≤39 and ≤43 cycles for the QF and VM qPCRs, respectively (data not shown). These results highlight the importance of evaluating and adapting the diagnostic cut-off values according to the epidemiological situation and, therefore, a previous verification of such thresholds should be performed before applying these methods in the field.

One of the main advantages of automated magnetic bead extraction protocols is their higher throughput capacity. However, the lower number of samples in certain epidemiological scenarios (i.e. bTB-free countries or low prevalence regions) may not compensate for the increased costs and use of reagents/plastics. Nevertheless, the KingFisher Flex script used for the purification of MTBC DNA can be used with other samples and infectious diseases, which would allow laboratories to easily combine samples in a single run, increasing the flexibility of diagnostic workflows. In addition, the decreased manipulation of columns and test tubes lowers the risk of cross-contamination.

Conclusion

In conclusion, the use of the MagMax CORE Nucleic Acid Purification kit coupled with the King Fisher Flex instrument could be an efficient alternative to the manual extraction using column-based kits in high throughput settings, allowing the scaling up of molecular diagnostic workflows in veterinary laboratories tasked with TB surveillance and control. The results of this study indicate that the IS*6110* qPCR recommended by the EU-RL for bTB and the VetMax™ *M. tuberculosis* Complex kit are viable approaches for the molecular diagnosis of TB in cattle, with high diagnostic performance and agreement. Nevertheless, the higher variability in Ct values in qPCR results from samples extracted using the magnetic

bead-based approach indicates that this method needs to be appropriately verified and adapted before its implementation in the routine diagnosis of bTB.

Methods

Sample selection

A total of 145 fresh bovine tissue samples were obtained from the routine diagnostic workflow performed by the VISAVET Centre for the diagnosis of bTB during the year 2023, following the guidelines of the National eradication programme for bTB of the Spanish government. Seventy-seven of these samples had visible lesions compatible with TB (VL), whereas 68 had no visible lesions (NVL).

Since late 2022, the majority of bTB samples processed in VISAVET are tested using the qPCR protocol (IS*6110*-based) and DNA extraction method (column-based) recommended by the EU-RL for bTB (https://www.visavet.es/bovinetuberculosis/databases/protocols.php) and, in general, only qPCR-positive samples are routinely tested using microbiological culture for epidemiological purposes. In order to compensate for any discrepancies in the results due to differences in the extraction methods, the true infection status of the sample was established using a composite reference standard based on the use of the EU-RL methods for DNA extraction and qPCR, as well as microbiological culture. An additional qPCR based on the IS*1081* element was used as an additional confirmation of IS*6110*-positive and culture-negative samples [25]. For this comparison and to ease the discussion, samples that were positive according to this composite reference standard were considered as "true positive samples".

Microbiological culture and DNA extraction

The initial DNA extraction and culture of the samples was performed in the BSL3 facilities at VISAVET as previously indicated [7, 8]. Briefly, fresh bovine lymph nodes were visually inspected for the presence of TB lesions and approximately two grams were cut and minced for their posterior homogenisation in 12 ml of PBS. Five ml of tissue homogenate were decontaminated using the same volume of 0.75% (w/v) hexadecyl pyridinium chloride solution in agitation for 30 min and centrifuged for the same amount of time at 1,300-1,500 g. The pellets were then collected using cotton swabs and seeded on Löwenstein-Jensen supplemented with sodium pyruvate and Coletsos solid media (Difco, Spain). Culture media were incubated at 37 °C for a maximum of 3 months or until growth compatible with TB was detected and confirmed using qPCR [7, 24].

For the DNA extraction, one and 1.8 ml of tissue homogenate were stored at -40 °C until processing for the column-based protocol (protocol COL) and the magnetic bead-based protocol (protocol KF), respectively. The COL protocol was performed using the DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany) including two mechanical lysis steps (3×40 s at 9,000 rpm) in a Precellys Evolution homogeniser (Bertin Instruments) with an overnight biochemical lysis step with proteinase K at 56 °C in between.

For the KF protocol, 1.8 ml of frozen tissue homogenate was extracted following the instructions of the VetMax™ *M. tuberculosis* Complex kit (Rev. E.0) and the manufacturers recommendations for the MagMAX™ CORE Nucleic Acid Purification kit (Applied Biosystems; Pub. No. MAN0015859 Rev. E.0). In this case, the homogenate was centrifuged, resuspended in 600 µl of 1X TE buffer (ThermoFisher Scientific) and added to a DNAse/RNAse-free tube containing 100 mg of 0.5 mm and 50 mg of 0.1 mm glass beads. The homogenates were then lysed on a Precellys Evolution instrument at 6,800 g for 3 cycles of 30 s and centrifuged. The supernatant was then transferred to a clean DNA/RNA-free tube and 200 µl were pipetted to a new tube containing 200 µl of MagMAX™ CORE Lysis Solution and 210 µl of Proteinase K solution and incubated 16–24 h at 56±4 °C. The lysate was then purified following the MagMAX_CORE_Flex_Express script on a KingFisher Flex instrument (ThermoFisher Scientific).

Real-time PCR

Samples extracted with either the COL or KF protocols were analysed using both the IS*6110* qPCR [24], recommended by the EU-RL and based on the QuantiFast Pathogen+IC kit (QIAGEN) (protocol QF), and the VetMAX™ *M. tuberculosis* Complex kit (protocol VM) [13]. Both protocols include an exogenous heterologous inhibition control (IC); in the case of the VM kit, the IC is included during the extraction, whereas the QF qPCR includes the IC in the qPCR master mix. Since the tissue samples were originally processed using the COL protocol, which does not include the IC for the VM kit, 1 µl of a 10^{-1} dilution of the VM IC was spiked in the VM qPCR master mix when analysing samples extracted using the COL protocol in order to obtain a cycle threshold (Ct) similar to the one indicated in the certificate of analysis of the lot used in the analysis (31±3). All qPCRs were performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and analysed in CFX Manager Maestro v2.3. All analyses were run on the same instrument. For this comparison, the diagnostic cut-off was set at 38 cycles for the QF qPCR [8] and 40 cycles for the VM qPCR, as recommended by the manufacturer. The maximum number of cycles on the VM qPCR was increased from 40 to 45 cycles.

Generation of spiked samples

In order to assess possible differences in the analytical properties of the MagMax CORE Nucleic Acid purification kit and the KingFisher Flex instrument, a standard curve of spiked bovine tissue samples was generated. *Mycobacterium bovis* BCG Danish was cultured on Middlebrook 7H9 media supplemented with sodium pyruvate (Difco, Madrid, Spain) and Oleic Albumin Dextrose Catalase (Becton Dickinson, Franklin Lakes, NJ). When confluent, the culture was set to 1 McFarland using a Densimat spectrophotometer (Biomerieux, Marcy-l'Étoile, France) and diluted 10-fold into the different aliquots obtaining a standard curve ranging from 10^6 to 10^3 UFC/ml [34]. A set of negative bovine tissues was homogenised in 12 ml as previously indicated, mixed and divided into four aliquots. Then, four aliquots of 1 ml and 1.8 ml of each dilution were stored at -40 °C until they were extracted as previously indicated using the COL and KF protocols, respectively. Negative tissue aliquots were also stored as negative extraction controls.

Statistical analyses

The PPA and NPA values of the qPCR analyses were calculated using MedCalc v22.021 (MedCalc, Ostend, Belgium), whereas concordance was calculated using Cohen's Unweighted Kappa in WinEpi 2.0 [35]. The agreement between the Ct values obtained by each combination of qPCR and DNA extraction protocol was evaluated through a Bland-Altman analysis [36], using the blandr package [37] in R version 4.2.3 [38]. Differences between average Ct values from the different extraction and qPCR protocol combinations were analysed using the t-test function, adjusting with the Holm-Bonferroni method.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04427-8.

Supplementary Material 1

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Author contributions

VL and BR designed the experiments. VL and AGT performed the experiments. VL analysed and interpreted the results. VL and AGB performed the statistical analyses. JB, LdJ and BR supervised the study. JB, LdJ and BR contributed to the funding acquisition. VL wrote the original draft and the remaining authors reviewed and approved the final manuscript.

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Data availability

The datasets of the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

All samples were obtained as part of the routine diagnosis workflow of the Spanish bTB eradication programme and, therefore, no animal was sacrificed for the specific purpose of this research study. No ethics approval is therefore required.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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