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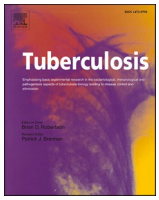

Review

Prevalence of *Mycobacterium bovis* in milk on dairy cattle farms: An international systematic literature review and meta-analysis

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ABSTRACT

Bovine tuberculosis, caused by *Mycobacterium bovis* (*M. bovis*), is a globally distributed chronic disease of animals. The bacteria can be transmitted to humans via the consumption of unpasteurised (raw) milk, thus representing an important public health risk. To investigate the risk of zoonotic transmission of *M. bovis* via raw milk, this study systematically reviewed published studies to estimate the prevalence of *M. bovis* in on-farm bulk-tank milk (BTM) and individual cow's milk (IM) by meta-analysis.

In total, 1,339 articles were identified through seven electronic databases and initially screened using titles and abstracts. The quality of 108 potentially relevant articles was assessed using full texts, and 67 articles comprising 83 studies (76 IM and 7 BTM), were included in the meta-analysis. The prevalence of *M. bovis* in IM and BTM was summarised according to the diagnostic test used, and the tuberculin skin test (TST) infection status of the individual cows (for IM) or herds (for BTM). Heterogeneity was quantified using the *I*-squared statistic. Prediction intervals (95% PIs) were also estimated.

For IM, the overall prevalence was summarised at 5% (95%CI: 3%–7%). In TST positive cows, prevalence was summarised at 8% (95%CI: 4%–13%). For BTM, the overall prevalence independent of individual herd TST infection status was summarised at 5% (95%CI: 0%–21%).

There was considerable heterogeneity evident among the included studies, while PIs were also wide. Inconsistency in the quality of reporting was also observed resulting in missing information, such as the TST infection status of the individual animal/herd. No study reported the number of *M. bovis* bacteria in test-positive milk samples. Several studies reported the detection of *M. tuberculosis* and *M. africanum* in milk.

Despite international efforts to control tuberculosis, this study highlights the risk of zoonotic transmission of *M. bovis* via unpasteurised milk and dairy products made using raw milk.

1. Introduction

Tuberculosis (TB) in humans is principally caused by the bacteria *Mycobacterium tuberculosis sensu stricto* and *Mycobacterium africanum*. Zoonotic tuberculosis is a form of TB in people that is typically associated with infection with *Mycobacterium bovis*, which belongs to the *M. tuberculosis* complex (MTBC). Other bacteria from the MTBC can also cause zoonotic TB, such as *M. caprae* and *M. orygis* (reviewed by Kock et al., 2021).

In 2019, 10 million incident cases of active TB in humans were estimated globally; among these, 140,000 (range 69,800–235,000) are estimated to be new cases of zoonotic TB (1.4%) of which approximately 11,400 (8.1%, range 4470–21,600) died [1,2]. In Ireland, an average of 6 incident cases of zoonotic tuberculosis (range 2–12) were notified annually between 2006 and 2018, accounting for 3.2% of all notified tuberculosis cases in 2018 [3]. It is not clear however, if these cases are autochthonous or imported, or whether there is a strong link between these (few) cases and the prevalence of bovine tuberculosis (bTB) in the Irish cattle herd.

Zoonotic tuberculosis incidence is higher in some regions and countries than others, particularly where there is a close association between number of cattle (the major source of *M. bovis*) and people

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(including many suffering from poverty), and where milk and dairy products are often consumed unpasteurised [1]. The burden of TB in animals varies considerably across countries and continents; this is consistent with differences in predominant livestock systems. The highest prevalence of animal disease is reported from the Americas and Europe [4], however the available data are likely to be biased due to different diagnostic capacities and sampling strategies used in different regions [1]. Risk factors for zoonotic tuberculosis include high human-animal density, consumption of unpasteurised milk and milk products, close and frequent physical contact between humans and infected animals and inadequate disease control measures [1,5,6]. The most common route of transmission of *M. bovis* to humans is through contaminated food, principally untreated dairy products or, less commonly, untreated meat products, although other routes (aerosol inhalation or direct contact with mucous membranes and skin abrasions) are also possible [7].

A recent scientific opinion on the public health risks relating to the consumption of raw drinking milk highlighted the risk of zoonotic tuberculosis associated with unpasteurised milk and milk products [8]. In the UK, there is an ongoing risk of *M. bovis* infection for some individuals due to continuing on-farm consumption of unpasteurised cow's milk, retail sales of unpasteurised milk and dairy products by approved establishments, and occupational exposure to infectious aerosols from tuberculous animals and their carcasses [7]. Outbreaks of zoonotic tuberculosis in developed countries are now rare but do occur. This is evidenced by an outbreak of *M. bovis* infection in people and cattle on a dairy farm in Ireland that was reported by Doran et al. (2009) [9], highlighting the ongoing risks associated with raw (unpasteurised) milk consumption.

To investigate human exposure to *M. bovis* via raw milk, information on the burden of *M. bovis* contamination at each level in the milk processing chain is necessary, starting at the level of the farm. This information can be used to help inform risk assessments relating to the potential risk of zoonotic tuberculosis from unpasteurised milk and dairy products made using raw milk. While several studies have investigated the occurrence of *M. bovis* in raw bovine milk samples at farm level in different regions, no study to date has synthesised these reports into a single systematic review and meta-analysis.

Hence, this study aims to estimate the prevalence of *M. bovis* in on-farm bulk tank milk (BTM) and individual cow's milk (IM) by systematic review and meta-analysis, and to investigate possible factors associated with the variation in the reported *M. bovis* prevalence across studies.

2. Materials and methods

2.1. Systematic literature review

2.1.1. Literature search

Seven electronic databases were searched for published articles: Science Direct, Embase, Web of Science, Scopus, Medline, CAB abstracts and PubMed (Fig. 1).

Search keywords used were: "Mycobacterium bovis", "bovine tuberculosis", "bovine tb", "M. bovis", "btb" each combined with "milk". Where available, subject headings were included for Mycobacterium bovis and milk. The search terms used for each database are provided in the supplementary file. No date on language restrictions were imposed at this stage. Bibliographies within publications were also searched for further relevant articles. Additionally, all available proceedings of the International Conference on Mycobacterium bovis (1st-6th; 1994–2014) were searched using the same keywords. The literature search included any study testing raw milk collected on farms, regardless of the specified study objectives, in order to maximize the sensitivity of the search.

Records were uploaded into a systematic review management software: Covidence (www.covidence.org). Duplicates were removed by matching the first author, title, and publication year.

2.1.2. Title and abstract screening

Title and abstracts were screened for relevance within Covidence using the following inclusion criteria: (1) raw milk samples collected on farm and tested for the presence of *M. bovis* or MTBC bacteria or DNA (2) primary research studies, (3) articles in English, and (4) articles published between 1970 and July 2021. Studies reported prior to 1970 were excluded because these studies were conducted before quantitative culture methods for *M. bovis* from dairy product samples had been robustly developed and the taxonomic status of *M. bovis* and

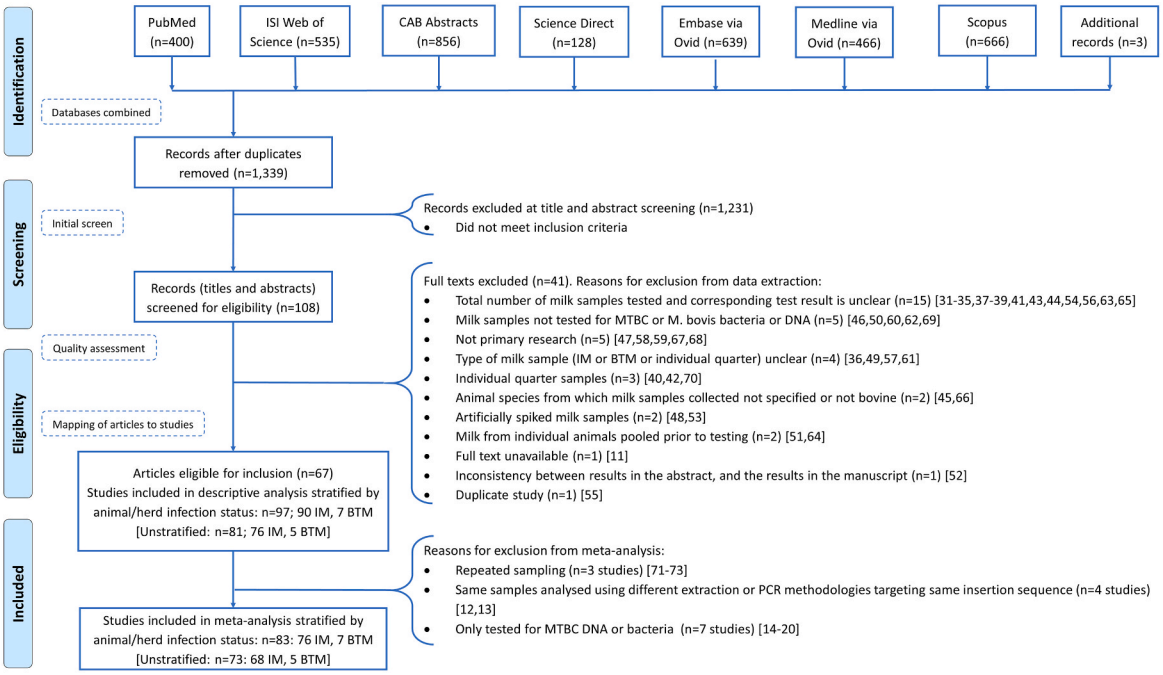

Fig. 1. Steps and results of the systematic review and meta-analysis for *Mycobacterium bovis* in milk (bulk tank milk: BTM and individual cow milk: IM) at farm level.

M. tuberculosis had been resolved [10]. When an abstract was not available and the relevance could not be determined from the title alone, the record was screened for inclusion in the full text review.

2.1.3. Full text review

Further inclusion of the articles was assessed using full texts. Full text articles were obtained for all potentially relevant articles except one [11].

The criteria considered essential for inclusion in the review were: description of the total number of samples tested and their corresponding test results, description of the milk sampling and description of the test method used. At this step, the articles were mapped to study level, i.e. the combination of milk samples (bulk tank milk; BTM, or individual cow's milk; IM), the test method used (culture only, PCR only or a combination of culture and PCR (culture first confirmed by PCR or *vice versa*)) and the cow/herd tuberculin skin test (TST) infection status (see Section 2.2.2 for details of this stratification).

Exclusion criteria were studies on animals other than cattle (e.g. camels, sheep, goats and buffalo), studies on pasteurised milk or dairy products other than raw liquid-milk (e.g. cheese, fermented/cultured milks), and studies testing spiked or artificially contaminated milk samples (Fig. 1).

2.1.4. Data extraction

A data extraction template was created in Microsoft Excel similar to one reported by [163]. Data extracted from the relevant studies included general information of the article, population characteristics, study design, measurement of the outcomes, statistical analyses, and results.

In the review, an individual milk (IM) sample referred to a sample collected from an individual cow on one test-day. The study unit for IM was an individual cow. Therefore, studies describing milk samples representing an individual teat of an individual cow were excluded. Further, studies where samples were collected from individual cows over multiple days (multiple samples collected from individual cows over multiple days) were included in the descriptive analysis but were excluded from the meta-analysis because samples collected from individual cows over multiple days are expected to be highly correlated with respect to the presence of *M. bovis*, given the chronic nature of the infection. A bulk tank milk (BTM) sample referred to a sample collected from the farm bulk tank of a dairy cow herd on a single day. Therefore, the study unit for BTM was an individual dairy herd. Repeat BTM sampling (multiple BTM samples collected from an individual herd over multiple days) was not an issue since the literature search did not identify any such studies.

Because members of the MTBC other than *M. bovis*, such as *M. tuberculosis* or *M. africanum*, can also cause infection and disease in humans, studies reporting the investigation of any member of the MTBC in milk were included in the descriptive tables. However, they were excluded from the subsequent meta-analysis for *M. bovis* in milk.

2.2. Data analysis

2.2.1. Descriptive analysis

The descriptive analysis of the extracted data was done by summarizing into tables, with stratification by milk sample type, cow/herd infection status and diagnostic test method used (further detail provided in Section 2.2.2 below). Several studies included in the descriptive tables were excluded from the subsequent meta-analysis in order to keep equal weight to each set of milk samples. Firstly, when a set of milk samples was analysed by PCR using different DNA extraction techniques (e.g. Cornejo et al., 1998 [12]) or different primers targeting the same target sequence or gene (e.g. Zumarraga et al., 2005 [13]), all were presented in the descriptive analysis; however only one (the method most closely aligned with the main objective of the article) was used for the statistical analysis. Secondly, studies which only reported the detection of MTBC bacteria or DNA (rather than *M. bovis*) were also excluded from the meta-analysis [14–20], because the objective of the meta-analysis was to estimate the prevalence of *M. bovis* in milk. Studies where all samples tested negative for MTBC bacteria or DNA were included in the meta-analysis because it was assumed that a sample testing negative for MTBC is also test negative for *M. bovis*.

2.2.2. Stratification

Milk samples collected on farms were characterised by three strata: type of milk sample (IM or BTM), diagnostic test method used for the detection of *M. bovis* in milk (culture only or PCR only or combined culture and PCR), and the TST infection status of the individual cow (for IM) or herd (for BTM).

Three types of diagnostic test methods were generally used to detect *M. bovis* in milk: detection of *M. bovis* bacteria by culture only, by PCR only, or by combined culture and PCR (culture first confirmed by PCR or *vice versa*). Studies where the detection of *M. bovis* in milk involved culture of bacteria on solid or liquid media only, were grouped into 'culture only'. Detection methods where the presence of *M. bovis* or MTBC involved the detection of DNA specific genes or insertion sequences only, were grouped into 'PCR only'. Studies which used a combination of both culture and PCR (culture first confirmed by PCR or *vice versa*), were grouped into 'combined culture and PCR'. There are further differences within the test methods in terms of practical procedures such as pre-enrichment and DNA extraction, but analysis of this information was considered beyond the scope of this present review.

Studies were grouped by the TST infection status of individual cows/herds because *M. bovis* shedding in milk depends on the TST infection status of the individual animal/herd. For example, TST positive cows are more likely to shed *M. bovis* in their milk than TST negative cows due to the chronic nature of bovine tuberculosis. Similarly, TST positive herds are more likely to have *M. bovis* present in the bulk tank milk compared with TST negative herds. Animals were grouped into 'positive' or 'negative' when they had a positive or negative tuberculin skin test (TST); either Single Intradermal Tuberculin (SIT) test, Single Intradermal Comparative Tuberculin test (SICTT) or Caudal Fold Tuberculin (CFT) test, prior to, or simultaneously with, the milk testing. Similarly, herds were grouped into 'positive' or 'negative' herds depending on the TST status of that herd. Studies with no information on the TST infection status of the source animals/herds, or where the individual cow/herd infection status corresponding to each milk sample could not be determined, were described with 'unknown' infection status.

2.2.3. Meta-analyses

A meta-analysis using a random-effects model was used to estimate the *M. bovis* prevalence in milk. Analyses were performed to summarise overall prevalence in IM (independent of the individual cow infection status) stratified by diagnostic test method group, as well as a sub-group analysis in TST positive cows only. Sub-group analysis in TST negative cows was not carried out because misclassification of the TST infection status of these cows was considered likely because TST negative cows typically originated in herds with a positive or unknown TST infection status (Tables 1–3). It is recognised that individual cows with a negative TST and originate in herds with a positive TST infection status have increased risk of infection with *M. bovis* given the chronic nature of the disease [21]. Further, given the documented variability in the sensitivity of the TST which can be as low as 26% [22], is it plausible that the TST infection status of several of these animals was misclassified due to false negative TST results.

A random-effects model was chosen because the true prevalence was assumed to vary across studies, where the reported prevalence reflects the true variation, random variation and differences in test characteristics [23]. The estimated prevalence and 95% confidence intervals (95%CI) were calculated for each diagnostic test method using random-effects meta-analyses for binomial data using the Stata Metaprop package [24]. The Metaprop command computes 95%CI using the score statistics (for smaller sample size) that allows incorporation of the

Table 1
Apparent prevalence of *Mycobacterium bovis* and *Mycobacterium tuberculosis* complex (MTBC) in individual cow's milk samples collected on cattle farms and tested by **culture** method, stratified by individual animal infection status (n = 30 studies).

Individual animal bTB infection status test	Culture media	Additional culture diagnostic tests	No. herds sampled	TST herd prevalence	No. samples tested	No. samples positive (Mycobacterium spp. identified)	*M. bovis* (MTBC) Apparent prevalence	Country	Study period	Authors
Individual animal infection status: Positive (n = 16)										
SIT	LJ, MB-7H10, MB-7H11	ZN, culture characteristics, biochemical tests	NR	NR	26	2 (2 *M. bovis*)	0.08 (0.08)	Egypt	2000–2001	(Abou-Eisha et al., 2002) [134]
SIT	LJ-P, LJ-G	ZN, culture characteristics, biochemical tests, guinea pig inoculation	NR	NR	105	5 (5 *M. bovis*)	0.05 (0.05)	Egypt	2008–2010	(Alwathnani et al., 2012) [135]
SIT, SICTT	LJ-P, LJ-G	No growth observed	NR	NR	24	0 (0 *M. bovis* 0 MTBC)	0.00 (0.00)	Ethiopia	2001–2002	(Ameni et al., 2003) [85]
SIT	LJ, LJ-P	ZN, culture characteristics, biochemical tests	NR	NR	91	4 (4 *M. bovis*)	0.04 (0.04)	Ethiopia	1999	(Asseged et al., 2000) [136]
SIT	LJ-G, ST	ZN, culture characteristics, biochemical tests	4	NR	270	15 (13 *M. bovis* 2 *M. tuberculosis)*	0.05 (0.06)	India	NR	(Aswathanarayana et al., 1998) [94]
SICTT	Culture method as described by [137]		NR	NR	150	38 (38 *M. bovis)*	0.25 (0.25)	Brazil	NR	(Carvalho et al., 2014) [17]
TST*[1]	Culture method as described by [138,139]		4	NR	17	4 (4 *M. bovis*	0.24 (0.24)	Mexico	NR	(Cornejo et al., 1998) [12]
SICTT	LJ, LJ-P	ZN, culture characteristics, biochemical tests	106	0.43 (46 positive herds)	141	6 (5 *M. bovis* 1 *M. tuberculosis)*	0.04 (0.04)	Ethiopia	2005–2006	(Elias et al., 2008) [86]
SIT	LJ	ZN, culture characteristics, biochemical tests	11	1.00 (11 positive herds)	215	5 (5 *M. bovis)*	0.02 (0.02)	Egypt	2017	(Elsohaby et al., 2020) [74]
SICTT	LJ-P	NR	NR	NR	16	1 (1 MTBC)	n/a (0.06)	Ethiopia	2007–2008	(Regassa et al., 2010) [14]*[2]
SIT	LJ-P, LJ-G	ZN, culture characteristics, biochemical tests	NR	NR	50	2 (2 *M. bovis)*	0.04 (0.04)	Egypt	NR	(Nasr et al., 2013) [140]
SIT, SICTT	LJ-P, LJ-G	ZN, culture characteristics, biochemical tests	NR	NR	24	3 (3 *M. bovis)*	0.13 (0.13)	Ethiopia	2007–2008	(Tigre et al., 2011) [141]
TST*[1]	LJ	ZN, culture characteristics	NR	NR	1285	33 (33 MTBC)	n/a (0.03)	Egypt	2018–2019	(Abdelsadek et al., 2020) [15]*[2]
SICTT, IFNγ	LJ-P, LJ-G	ZN	1	1.00 (1 positive herd)	23	9 (9 MTBC)	n/a (0.39)	Ethiopia	2004	(Lambert et al., 2006) [16]*[2]
Individual animal infection status: Positive (n = 16)										
SICTT	LJ-P, LJ-G	ZN, culture characteristics, biochemical tests	NR	NR	72	7 (5 *M. bovis* 2 *M. tuberculosis)*	0.07 (0.10)	Ethiopia	2007–2008	(Fetene et al., 2011) [95]
SICTT	LJ	ZN, culture characteristics, thin layer chromatography	6	NR	780	1 (1 *M. bovis)*	0.10 (0.10)	Brazil	1998	(Pardo et al., 2001) [73]*[3]
Individual animal infection status: Negative (n = 3)										
SIT	LJ-P, LJ-G	ZN, culture characteristics, biochemical tests, guinea pig inoculation	NR	NR	125	2 (2 *M. bovis)*	0.02 (0.02)	Egypt	2008–2010	(Alwathnani et al., 2012) [135]
SIT	LJ-P, LJ-G	ZN, culture characteristics, biochemical tests	NR	NR	50	1 (1 *M. bovis)*	0.02 (0.02)	Egypt	NR	(Nasr et al., 2013) [140]
SICTT	ST, Sula	No growth observed	1	1.00 (1 positive herd)	15	0 (0 *M. bovis* 0 MTBC*)*	0.00 (0.00)	Czech Republic	1995	(Pavlik et al., 2002) [142]*[4]
Individual animal infection status: Unknown or not reported (n = 11)										
SCT, CFT	LJ-P, LJ-G	ZN, culture characteristics, biochemical tests	1	1.00 (1 positive herd)	50	19 (19 *M. bovis)*	0.38 (0.38)	Egypt	2005	(Ghazy et al., 2007) [143]
SIT, CFT	LJ-P, LJ-G	Culture characteristics, biochemical tests	1	1.00 (1 positive herd)	23	1 (1 *M. bovis)*	0.04 (0.04)	Egypt	NR	(Hassanain et al., 2009) [144]
SIT, SICTT	ST	ZN	5	NR	200		0.00 (0.00)	Colombia	NR	

(*continued on next page*)

Table 1 (*continued*)

Individual animal bTB infection status test	Culture media	Additional culture diagnostic tests	No. herds sampled	TST herd prevalence	No. samples tested	No. samples positive (Mycobacterium spp. identified)	*M. bovis* (MTBC) Apparent prevalence	Country	Study period	Authors
						0 (0 *M. bovis* 0 MTBC)				(Romero et al., 1999) [76]
SICTT	LJ-P, LJ-G	ZN, culture characteristics, biochemical tests	NR	NR	154	10 (6 *M. bovis* 4 *M. tuberculosis)*	0.04 (0.06)	India	1999–2000	(Srivastava et al., 2008) [96]
–	LJ-P	ZN, culture characteristics, biochemical tests	NR	NR	68	7 (7 *M. bovis)*	0.10 (0.10)	Iraq	NR	(Al-Thwani et al., 2015) [145]
–	LJ-G, LJ-P	NR	NR	NR	181	1 (0 *M. bovis* 1 *M. tuberculosis)*	0.00 (0.01)	India	NR	(BhanuRekha et al., 2015) [97]
–	MB-7H11	ZN, culture characteristics, biochemical tests, spoligotyping	NR	NR	53	6 (6 *M. bovis)*	0.11 (0.11)	Nigeria	NR	(Cadmus and Adesokan, 2007) [81]
–	LJ, LJ-G, ST-pyruvate	ZN, culture characteristics, biochemical tests, rabbit inoculation	NR	NR	757	6 (6 *M. bovis)*	0.01 (0.01)	Egypt	NR	(Guindi et al., 1980) [101]
–	LJ-P, LJ-G	ZN, culture characteristics, biochemical tests	5	NR	285	4 (4 *M. bovis)*	0.01 (0.01)	Nigeria	2005–2006	(Ofukwu et al., 2008) [72]*[3]
–	LJ, LJ-P, LJ-G	ZN, culture characteristics, biochemical tests, rabbit inoculation	NR	NR	400	26 (24 *M. bovis* 2 *M. tuberculosis)*	0.06 (0.07)	Nigeria	NR	(Okolo, 1992) [98]
–	LJ-P, LJ-G	ZN, culture characteristics, guinea pig inoculation	13	NR	150	8 (8 *M. bovis)*	0.05 (0.05)	Egypt	2016	(Sarah et al., 2019) [146]

bTB: bovine tuberculosis; **CFT**: Caudal fold test; **ELISA**: Enzyme linked immunosorbent assay; **IFNγ**: interferon gamma assay; **LJ**: Löwenstein–Jensen; **LJ-G**: Löwenstein–Jensen with glycerin/glycerol; **LJ-P**: Löwenstein–Jensen with pyruvate; MB: Middlebrook; **NR**: Not reported; **SICTT**: Single intradermal comparative cervical tuberculin skin test; **SIT**: Single intradermal cervical tuberculin skin test; **ST**: Stonebrinks; **TST**: Tuberculin Skin Test; **ZN**: Ziehl–Neelsen staining.
*1: Precise tuberculin skin test not specified; *2: Excluded from meta-analysis because only tested for MTBC, *3: Excluded from meta-analysis due to multiple (repeat) sampling; *4: Milk samples collected from tuberculin skin test negative cows in a positive herd.

Freeman-Tukey double arcsine transformation [25] of proportions to generate admissible pooled estimates and 95%CIs that must fall within the range of [0.00–1.00].

Heterogeneity was quantified using the I-squared (I^2) statistic [26,27]; this describes the percentage of the variability that is due to heterogeneity in the true prevalence among studies rather than due to sampling error, with higher percentages indicating higher heterogeneity [28,29]. Study-level characteristics were explored in order to investigate the sources of heterogeneity among studies, however meta-regression was not possible due to inconsistency in the quality of reporting across studies and missing information (further details provided below).

Additionally, 95% predictive intervals (95% PI) were estimated; in contrast to I^2, the 95% PI estimates the interval within which a future observation (study) will fall 95% of the time, based on the studies included in the present meta-analysis [30]. Hence, prediction intervals (PIs) represent the uncertainty of predicting the value of a single future observation (study).

3. Results

3.1. Systematic literature review

3.1.1. Identification and description of relevant literature

Initially, 1,339 articles were identified from the literature searches. After the first screening, 108 articles passed the title and abstract screening eligibility assessment. In the full text review, 41 articles were excluded prior to data extraction for reasons given in Fig. 1 [11,31–70].

As a result, the descriptive tables included 90 individual cow milk studies (Tables 1–3) and 7 bulk tank milk studies (Table 4) stratified by individual cow or herd TST infection status, respectively.

3.1.2. Diagnostic test characteristics and possible M. bovis load in test positive milk samples

Characteristics of the test used to analyse the milk samples (e.g. test sensitivity and specificity, use of positive/negative controls, detection limit) was rarely reported.

Among the 97 studies included in the descriptive tables, nine studies from five articles reported the diagnostic test sensitivity and/or specificity for PCR [12,74–78], while only one study reported the culture test sensitivity and specificity [74].

Seven PCR studies from four articles [13,17,75,79] reported a detection limit for PCR at 100 colony-forming bacterial units (CFU)/mL, 200 CFU/mL, 3 CFU/mL, and 3 CFU/mL, respectively (Tables 1–4). No study reported the detection limit for culture. Garbaccio et al. (2018) reported "*The detection limit of the bacteriological test must also be considered, in which values higher than 10 or 100 viable microorganisms are required to obtain a positive result*". Similarly, Abdelsadek et al. (2020) noted that "*Culture methods may detect as few as 10^1-10^2 organisms/m[L] in a single specimen*" [15].

Thirty-seven studies (37/97; 38%) reported using controls; 24 studies using PCR reported the use of both positive and negative controls, and 12 reported using either a positive or negative control. Only two studies using culture methods (culture only, or culture with PCR) reported the use of a culture control; Cadmus and Adesokan, (2007) [81] (negative control) and Bolaños et al. (2018) [82] (positive control).

No study provided an estimate for the number of *M. bovis* bacteria (e.g. the number of CFU/mL) in milk. Among the studies excluded at full text review, Mariam (2014) reported the detection and quantification of *M. tuberculosis* in a pooled milk sample obtained from 30 TST positive cows on a farm in Ethiopia; the authors reported 4.7 ± 4.4 log CFU/mL in the pooled milk sample [64].

Table 2
Apparent prevalence of *Mycobacterium bovis* and *Mycobacterium tuberculosis* complex (MTBC) in individual cow's milk samples collected on cattle farms and tested by **PCR** method, stratified by individual animal infection status (n = 35 studies).

Individual animal bTB infection status test	PCR method	Target gene/ sequence	No. herds sampled	TST herd prevalence	No. samples tested	No. samples positive (Mycobacterium spp. identified)	*M. bovis* (MTBC) Apparent prevalence	Country	Study period	Authors
Individual animal infection status: Positive (n = 12)										
SIT	N-PCR	16SrRNA, RvD1Rv2031c	NR	NR	105	8 (6 *M. bovis* 2 MTBC)	0.06 (0.08)	Egypt	2008–2010	(Alwathnani et al., 2012) [135]
SICTT	PCR	IS6110	NR	NR	150	75 (75 MTBC)	n/a (0.50)	Brazil	NR	(Carvalho et al., 2014) [17]*[2,3]
TST*[1]	PCR	IS6110	4	NR	17	10 (10 *M. bovis*)	0.59 (0.59)	Mexico	NR	(Cornejo et al., 1998) [12]*[4]
TST*[1]	PCR	IS6110	4	NR	17	16 (16 *M. bovis*)	0.94 (0.94)	Mexico	NR	(Cornejo et al., 1998) [12]
SICTT	PCR	16SrRNA	NR	NR	42	0 (0 MTBC 0 *M. bovis*)	0.00 (0.00)	Tanzania	2005	(Durnez et al., 2009) [147]
SIT	PCR	mpb70	11	1.00 (11 positive herds)	215	12 (12 *M. bovis*)	0.06 (0.06)	Egypt	2017	(Elsohaby et al., 2020) [74]
SICTT	PCR	RD4, RD9	3	NR	230	0 (0 MTBC 0 *M. bovis*)	0.00 (0.00)	Sri Lanka	2016–2017	(Jayasumana et al., 2018) [79]*[5]
CFT, IFNγ	N-PCR	mpb70	1	1.00 (1 positive herd)	21	6 (6 *M. bovis*)	0.29 (0.29)	Mexico	NR	(Serrano-Moreno et al., 2008) [148]
SIT	PCR	hupB	NR	NR	17	3 (3 *M. bovis*)	0.18 (0.18)	India	NR	(Sharma et al., 2019) [149]
SIT	PCR	IS6110, Rv1506c (RD4)	3	0.67 (2 positive herds)	54	5 (5 *M. bovis*)	0.09 (0.09)	India	NR	(Thakur et al., 2016) [78]
SIT, SICTT, IFNγ, ELISA	PCR	IS1561, RD4	8	1.00 (8 positive herds)	46	39 (39 *M. bovis*)	0.85 (0.85)	China	NR	(Xu et al., 2021) [93]
SIT	PCR	IS6110	1	1.00 (1 positive herd)	20	3 (3 MTBC)	n/a (0.15)	India	NR	(Sreedevi and Krishnappa, 2003) [18]*[2]
Individual animal infection status: Negative (n = 5)										
SIT	N-PCR	16SrRNA, RvD1Rv2031c	NR	NR	125	4 (4 *M. bovis*)	0.03 (0.03)	Egypt	2008–2010	(Alwathnani et al., 2012) [135]
SICTT	PCR	RD4, RD9	3	NR	100	0 (0 MTBC 0 *M. bovis*)	0.00 (0.00)	Sri Lanka	2016–2017	(Jayasumana et al., 2018) [79]*[5]
CFT, IFNγ	N-PCR	mpb70	1	1.00 (1 positive herd)	23	2 (2 *M. bovis*)	0.09 (0.09)	Mexico	NR	(Serrano-Moreno et al., 2008) [148]*[6]
SICTT	PCR	IS6110, RvD1Rv2031c	1	1.00 (1 positive herd)	8	5 (5 *M. bovis*)	0.63 (0.63)	Brazil	NR	(Zarden et al., 2013) [150]*[6]
SIT	PCR	IS6110	1	1.00 (1 positive herd)	1	0 (0 MTBC 0 *M. bovis*)	0.00 (0.00)	India	NR	(Sreedevi and Krishnappa, 2003) [18]*[5]
Individual animal infection status: Unknown or not reported (n = 18)										
SIT, SICTT	PCR	16SrRNA, RD9, RvD1Rv2031c	9	NR	96	4 (4 *M. bovis*)	0.04 (0.04)	India	NR	(Das et al., 2018) [130]
SICTT	PCR	16SrRNA	26	NR	226	0 (0 MTBC 0 *M. bovis*)	0.00 (0.00)	Tanzania	2005–2006	(Durnez et al., 2011) [151]
SIT, SICTT	PCR	RvD1Rv2031c	5	NR	200	4 (4 *M. bovis*)	0.02 (0.02)	Colombia	NR	(Romero et al., 1999) [76]
SICTT	PCR	IS6110	52	NR	146	1 (1 MTBC)	n/a (0.01)	Turkey	2005	(Solmaz et al., 2009) [19]*[2]
NR	PCR	pncA, RvD1Rv2031c	NR	NR	62	4 (4 *M. bovis* 0 *M. tuberculosis*)	0.06 (0.06)	Pakistan	NR	(Basit et al., 2018) [91]
NR	M-PCR	IS6110, Rv1506c (RD4)	NR	NR	181	4 (0 *M. bovis* 4 *M. tuberculosis*)	0.00 (0.02)	India	NR	(BhanuRekha et al., 2015) [97]
NR	PCR	RD4	20	NR	401	1 (1 *M. bovis*)	0.00 (0.00)	Brazil	2014	(Cezar et al., 2016) [152]
NR	PCR	IS6110, oxyR	1	NR	30	0 (0 *M. bovis* 0 MTBC)	0.00 (0.00)	West bank	2010	(Ereqat et al., 2013) [153]
NR	N-PCR	IS6110	NR	NR	50	9 (8 *M. bovis* 1 *M. tuberculosis*)	0.18 (0.16)	Nigeria	NR	(Ogundeji et al., 2015) [99]
NR	PCR	RD1	13	NR	150	8 (8 *M. bovis*)	0.05 (0.05)	Egypt	2016	

(*continued on next page*)

Table 2 (*continued*)

Individual animal bTB infection status test	PCR method	Target gene/ sequence	No. herds sampled	TST herd prevalence	No. samples tested	No. samples positive (Mycobacterium spp. identified)	*M. bovis* (MTBC) Apparent prevalence	Country	Study period	Authors
										(Sarah et al., 2019) [146]
NR	PCR	IS6110	NR	NR	82	10 (10 *M. bovis*)	0.12 (0.12)	Iraq	NR	(Senthil et al., 2014) [77]
NR	PCR	RvD1Rv2031c	NR	NR	145	2 (2 *M. bovis*)	0.01 (0.01)	Nigeria	NR	(Usman et al., 2016) [84]
NR	TD-PCR	IS6110	1	1.00 (1 positive herd)	53	15 (15 *M. bovis*)	0.28 (0.28)	Argentina	NR	(Zumarraga et al., 2005) [13]*7
NR	PCR	IS6110	1	1.00 (1 positive herd)	53	12 (12 *M. bovis*)	0.23 (0.23)	Argentina	NR	(Zumarraga et al., 2005) [13]*8,9
NR	PCR	IS6110	1	1.00 (1 positive herd)	53	8 (8 *M. bovis*)	0.15 (0.15)	Argentina	NR	(Zumarraga et al., 2005) [13]*8,10
NR	PCR	IS6110	1	1.00 (1 positive herd)	53	0 (0 MTBC 0 *M. bovis*)	0.00 (0.00)	Argentina	NR	(Zumarraga et al., 2005) [13]*8,11
NR	TD-PCR	IS6110	1	0 (1 negative herd)	34	0 (0 MTBC 0 *M. bovis*)	0.00 (0.00)	Argentina	NR	(Zumarraga et al., 2005) [13]*7,12
NR	PCR	IS6110, mpb64	1	NR	200	11 (11 MTBC)	n/a (0.06)	South Africa	NR	(Silaigwana et al., 2012) [20]*2

bTB: bovine tuberculosis; **CFT**: Caudal fold test; **ELISA**: Enzyme linked immunosorbent assay; **IFNγ**: interferon gamma assay; **LAMP**: loop mediated isothermal amplification; **M-PCR**; Multiplex PCR; **N-PCR**: nested PCR; **NR**: Not reported; **SICTT**: Single intradermal comparative cervical tuberculin skin test; **SIT**: Single intradermal cervical tuberculin skin test; **TD-PCR**: Touchdown PCR; **TST**: Tuberculin Skin Test; **VNTR**: Variable Number of Tandem Repeats; **ZN**: Ziehl–Neelsen staining.
*1: Precise tuberculin skin test not specified; *2: Excluded from meta-analysis because only tested for MTBC; *3: Reported detection limit 100 CFU/mL; *4: Excluded from meta-analysis because same samples were analysed using different PCR extraction technique; *5: Reported detection limit 200 CFU/mL; *6: Milk samples collected from tuberculin skin test negative cows in a positive herd; *7: Reported detection limit 3 CFU/mL, INS1 and INS2 primers; *8: Excluded from meta-analysis because same samples were analysed using different PCR methodologies; *9: 38-cycle conventional PCR with INS1 and INS2 primers.
*10: 30-cycle conventional PCR with IS1 and IS2 primers; *11: 30-cycle conventional PCR with INS1 and INS2 primers; *12: Milk samples collected from individual cows with unreported tuberculin skin test status in a negative herd.

3.1.3. Study design and population characteristics

Only three authors reported sample size calculations to estimate the prevalence of *M. bovis* in bovine milk; da Silva Cezar et al. (2016) [83] studied the prevalence of *M. bovis* in dairy cattle in State of Pernambuco, Brazil with a sample size of 385 and Bolaños et al. (2018) [82] studied the prevalence of mycobacteria in milk from SICCT positive cows in the State of Paraná, Brazil with a sample size of 142. The number of samples obtained by Usman et al. (2017) [84] was 40 samples short of the 185 samples required to estimate the prevalence of *M. bovis* in bovine milk in Bwari Area Council, Nigeria to the desired level of precision.

Sampling strategies used to collect milk samples were rarely described, and when they were, the quality of reporting was poor. Most articles (56/67; 84%) [studies (79/90; 88%)] appeared not to use simple random sampling or cluster-based random sampling methods. Ten IM studies and one BTM study reported probability sampling strategies including clustered sampling [72,85,86] and systematic sampling [72,87,88]. Five authors reported "random" sample collection, but did not adequately describe the sampling strategy used [19,89–92].

In the selected studies, the TST infection status of the individual cow or herd was frequently either not reported or was unclear. Among the IM studies, this was the case in 11 (37%) of the 30 studies that conducted culture only (Table 1), 18 (51%) of the 35 studies that undertook PCR only (Table 2) and 11 (44%) of the 25 studies that conducted combined culture and PCR (Table 3). Similarly, with the 4 BTM articles, only one [71] recorded the herd infection status (Table 4). Further, the number of herds from which IM samples were collected was infrequently reported (50/90; 56%). Of note, milk samples collected from individual cows with a negative TST typically originated in TST positive herds or herds of unknown infection status. Only one study reported the stage of bovine tuberculosis infection in individual cows or herds; Xu et al. (2021) [93] examined milk samples for *M. bovis* from individual cows with advanced disease (defined as: "*In serial testing, positive results from two or more assays (including ELISA) were considered as advanced infection*") in China.

3.2. Meta-analyses

Of the 90 IM studies included in the descriptive tables, three were excluded from the meta-analysis due to multiple (repeat) sampling [71–73], four were excluded because samples were tested for the same target sequence using different PCR primers/extraction technique [12,13] and seven were excluded because they only reported the detection of MTBC bacteria or DNA rather than *M. bovis* [14–20] (Tables 1–4, Fig. 1). Of the seven BTM studies, only Zumarraga et al. (2012) [75] reported the infection status of the sampled herds [75]; hence the BTM meta-analysis was not stratified by herd infection status (reported independent of herd infection status).

3.2.1. Prevalence of M. bovis in individual milk

The summarised prevalence for *M. bovis* in IM independent of individual cow infection status, stratified by diagnostic test method group is presented in Fig. 2. The prevalence estimate ranged between 0 and 94%, and was greatest in PCR only studies, followed by culture only studies and then combined culture and PCR studies, respectively. For each diagnostic test method, confidence intervals for the results of individual studies had little overlap indicating statistical heterogeneity among studies in the same diagnostic test method group ($p < 0.01$). Further, the I^2-statistic exceeded 89% in all three diagnostic test method groups, suggesting a large proportion of the variability in prevalence is due to heterogeneity in prevalence among study populations rather than sampling error (chance). There was also some evidence of heterogeneity between diagnostic test method groups ($p = 0.04$). Prediction intervals were also wide highlighting the uncertainty of predicting the value of a

Table 3
Apparent prevalence of *Mycobacterium bovis* and *Mycobacterium tuberculosis* complex (MTBC) in individual cow milk samples collected on cattle farms and tested using **combined culture and PCR**, stratified by individual animal infection status (n = 25 studies).

Individual animal bTB infection status test	Culture media	Additional culture diagnostic tests	PCR method	PCR target sequence and molecular typing method	No. herds sampled	TST herd prevalence	No. samples tested	No. samples positive (Mycobacterium spp. identified)	*M. bovis* (MTBC) Apparent prevalence	Country	Study period	Authors
Individual animal infection status: Positive (n = 12)												
SICTT	LJ, Colestos	ZN, culture characteristics	PCR,	IS6110, RD4; VNTR-typing, spoligotyping	9	1.00 (9 positive herds)	306	6 (6 *M. bovis)*	0.02 (0.02)	Tunisia	2005–2006	(Ben Kahla et al., 2011) [71]*[1]
SICTT	LJ, ST	ZN, culture characteristics	PCR	hsp65	NR	NR	142	0 (0 *M. bovis* 0 MTBC)	0.00 (0.00)	Brazil	2014–2015	(Bolaños et al., 2018) [82]
SICTT	LJ, ST, Ogawa-mycobactin, LJ-mycobactin	NR	PCR	16SrRNA	NR	NR	42	0 (0 *M. bovis* 0 MTBC)	0.00 (0.00)	Tanzania	2005	(Durnez et al., 2009) [147]
TST*[2]	LJ, ST	ZN	PCR,	IS6110; spoligotyping	24	NR*[3]	214	23 (23 *M. bovis)*	0.11 (0.11)	Argentina	2005–2012	(Garbaccio et al., 2018) [80]
SICTT	LJ, LJ-P	NR	PCR	RD4, RD9	3	NR	230	0 (0 *M. bovis* 0 MTBC)	0.00 (0.00)	Sri Lanka	2016–2017	(Jayasumana et al., 2018) [79]
SIT	7H9 liquid culture, Tween egg media	ZN, culture characteristics	M-PCR	rpoB, Rv1506c (RD4)	NR	NR	32	4 (4 *M. bovis)*	0.13 (0.13)	Nepal	2003	(Jha et al., 2007) [154]
TST*[2], IFNγ	LJ	ZN	D-PCR	IS6110, RvD1Rv2031c	NR	NR	7	0 (0 *M. bovis* 0 MTBC)	0.00 (0.00)	India	NR	(Neeraja et al., 2014) [155]
SICTT	LJ-P, LJ-G	ZN, culture characteristics, biochemical tests; nitrate/niacin	M-PCR, LAMP	cfp32, RD9, RD12	NR	NR	16	3 (3 *M. bovis)*	0.19 (0.19)	Zambia	2011–2012	(Pandey et al., 2013) [131]
SICTT	LJ, ST	ZN, culture characteristics	M-PCR	16SrRNA, mpb710, RD4	7	NR	16	3 (3 *M. bovis)*	0.19 (0.19)	Brazil	NR	(Ramos et al., 2016) [129]
SIT	LJ-P, LJ-G	ZN	M-PCR	IS6110, Rv1506c (RD4)	3	0.67 (2 positive herds)	54	0 (0 *M. bovis* 0 MTBC)	0.00 (0.00)	India	NR	(Thakur et al., 2016) [78]
SIT, SICTT, IFN-γ, ELISA	LJ, LJ-P	NR	PCR	IS1561, RD4	8	1.00 (8 positive herds)	46	10 (10 *M. bovis)*	0.22 (0.22)	China	NR	(Xu et al., 2021) [93]
SICTT	LJ-P, LJ-G	ZN	M-PCR	16SrRNA, mpb70	45	0.20 (9 positive herds)	55	0 (0 *M. bovis* 0 MTBC)	0.00 (0.00)	Ethiopia	NR	(Biru et al., 2014) [156]
Individual animal infection status: Negative (n = 2)												
SICTT	LJ, LJ-P	NR	PCR	RD4, RD9	3	NR	100	0 (0 *M. bovis* 0 MTBC)	0.00 (0.00)	Sri Lanka	2016–2017	(Jayasumana et al., 2018) [79]
SICTT	LJ-P	NR	M-PCR	IS6110, RvD1Rv2031c	1	1 (1 positive herd)	8	1 (1 *M. bovis)*	0.13 (0.13)	Brazil	NR	(Zarden et al., 2013) [150]*[5]
Individual animal infection status: Unknown or not reported (n = 11)												
SIT, SICTT	LJ-P, LJ-G	ZN, culture characteristics	PCR	16SrRNA, RD9, RvD1Rv2031c	9	NR	96	2 (2 *M. bovis)*	0.02 (0.02)	India	NR	(Das et al., 2018) [130]
SIT	LJ-P, LJ-G, MB-7H10, MGIT™	ZN, culture characteristics	N-PCR	IS6110, gryB; spoligotyping	8	NR	405	4 (0 *M. bovis* 4 *M. tuberculosis)*	0.00 (0.01)	India	2010–2015	(Mukherjee et al., 2018) [5]
SIT	ST	ZN, culture characteristics, biochemical tests	PCR	RvD1Rv2031c	NR	NR	1000	31 (31 *M. bovis)*	0.03 (0.03)	Pakistan	2007–2009	(Tipu et al., 2012) [89]*[4]
SICTT	ST		PCR	RvD1Rv2031c	NR	NR	793	31 (31 *M. bovis)*	0.04 (0.04)	Pakistan	NR	

(*continued on next page*)

Table 3 (*continued*)

Individual animal bTB infection status test	Culture media	Additional culture diagnostic tests	PCR method	PCR target sequence and molecular typing method	No. herds sampled	TST herd prevalence	No. samples tested	No. samples positive (Mycobacterium spp. identified)	*M. bovis* (MTBC) Apparent prevalence	Country	Study period	Authors
		ZN, culture characteristics										(Ullah et al., 2020) [90]*4
NR	LJ-P, LJ-G	NR	PCR	RD1, RD4, RD9, RD12; MIRU-VNTR, spoligotyping	NR	NR	144	2 (0 *M. bovis* 2 *M. tuberculosis)*	0.00 (0.01)	Nigeria	NR	(Adesokan et al., 2019) [100]
NR	LJ-P	ZN, culture characteristics, biochemical tests, rabbit inoculation	PCR	NR	NR	NR	93	8 (8 *M. bovis)*	0.09 (0.09)	Iraq	NR	(Al-Saqur et al., 2016) [157]
NR	MB-7H11	NR	PCR	RD1, RD4, RD9, RD12	40	0.10 (4 positive herds)	400	5 (4 *M. bovis* 1 *M. africanum)*	0.01 (0.01)	Nigeria	NR	(Cadmus et al., 2010) [87]
NR	IUT glycerol egg medium, LJ-P	ZN, culture characteristics, biochemical tests	PCR	IS986, mtp40	NR	NR	805	2 2 *M. bovis)*	0.00 (0.00)	Tanzania	NR	(Kazwala et al., 1998) [158]
NR	LJ, Colestos	ZN, culture characteristics, biochemical tests	PCR	RvD1Rv2031c	7	NR	300	37 (37 *M. bovis)*	0.12 (0.12)	Bang-ladesh	NR	(Rahman et al., 2015) [159]*4
NR	LJ-P	NR	M-PCR	RD4, RD9	NR	NR	30	2 (2 *M. bovis)*	0.07 (0.07)	South Africa	2017	(Sichewo et al., 2019) [160]
NR	LJ-P, LJ-G	ZN, culture characteristics	PCR	RvD1	NR	NR	145	2 (2 *M. bovis)*	0.01 (0.01)	Nigeria	NR	(Usman et al., 2016) [84]

bTB: bovine tuberculosis; **D-PCR**: duplex PCR; **ELISA**: Enzyme linked immunosorbent assay; **IFNγ**: interferon gamma assay; **IUT**: International Union Against Tuberculosis formulation of solid egg medium; **LAMP**: loop mediated isothermal amplification; **LJ**: Löwenstein–Jensen; **LJ-G**: Löwenstein–Jensen with glycerin/glycerol; **LJ-P**: Löwenstein–Jensen with pyruvate; **MB**: Middlebrook; **MGIT**: Commercial liquid medium Mycobacterial Growth Indicator Tube; **MIRU**: *Mycobacterium tuberculosis*-specific multiple locus; **M-PCR**; multiplex PCR; **N-PCR**: nested PCR.

NR: Not reported; **SICTT**: Single intradermal comparative cervical tuberculin skin test; **SIT**: Single intradermal cervical tuberculin skin test; **ST**: Stonebrinks; **TST**: Tuberculin Skin Test; **VNTR**: Variable Number of Tandem Repeats; **ZN**: Ziehl–Neelsen staining.

*1: Excluded from meta-analysis due to multiple (repeat) sampling; *2: Precise tuberculin skin test not specified; *3: The authors report "These rodeos presented an apparent prevalence that ranged between 0.5 and 4%"; *4: PCR first, confirmed by culture; *5: Milk samples collected from tuberculin skin test negative cows in a positive herd.

Table 4
Prevalence of *Mycobacterium bovis* (*M. bovis*) and *Mycobacterium tuberculosis* complex (MTBC) in **bulk tank milk** on cattle farms, stratified by herd bTB infection status (n = 7 studies).

Herd bTB infection status test used	Herd bTB infection status	Test method used	Culture media	Additional culture diagnostic tests	PCR method	Target gene/ sequence	No. samples tested	No. samples positive (Mycobacterium spp. identified)	*M. bovis* (MTBC) prevalence	Country	Study period	Authors
NR	NR	Culture	LJ, LJ-G, ST-pyruvate	ZN, culture characteristics, biochemical tests, rabbit inoculation	–	–	302	16 (10 *M. bovis* 6 *M. tuberculosis)*	0.03 (0.05)	Egypt	NR	(Guindi et al., 1980) [101]
TST*[1]	Negative*[2]	Culture	LJ, ST	No growth observed	–	–	177	0 (0 *M. bovis* 0 MTBC)	0.00 (0.00)	Argentina	2003–2008	(Zumarraga et al., 2012) [75]
TST*[1]	Positive*[3]	Culture	LJ, ST	No growth observed	–	–	80	0 (0 *M. bovis* 0 MTBC)	0.00 (0.00)	Argentina	2003–2008	(Zumarraga et al., 2012) [75]
TST*[1]	Negative*[2]	PCR	–	–	PCR	IS6110	177	67 (67 *M. bovis)*	0.38 (0.38)	Argentina	2003–2008	(Zumarraga et al., 2012) [75]*[4]
TST*[1]	Positive*[3]	PCR	–	–	PCR	IS6110	80	35 (35 *M. bovis)*	0.44 (0.44)	Argentina	2003–2008	(Zumarraga et al., 2012) [75]*[4]
NR	NR	Combined culture and PCR *[5]	LJ, ST	ZN, culture characteristics	RFLP-PCR	hsp65, gyrB	100	1 (1 *M. bovis)*	0.01 (0.01)	Brazil	NR	(Junqueira Franco et al., 2013) [161]
NR	NR	Combined culture and PCR *[5]	LJ	NR	PCR	IS6110, oxyR, mpb70	100	1 (1 *M. bovis)*	0.01 (0.01)	Egypt	2013	(El-Gedawy et al., 2014) [162]

bTB: bovine tuberculosis; BTM: Bulk tank milk; LJ: Löwenstein–Jensen; LJ-G: Löwenstein–Jensen with glycerin/glycerol; NR: not reported; RFLP: Restriction Fragment Length Polymorphism.
ST: Stonebrinks; ZN: Ziehl–Neelsen staining; TST: Tuberculin skin test.
***1**: Precise tuberculin skin test not specified; ***2**: Official tuberculosis free certificate (TFC); ***3**: Non-tuberculosis free certificate (NTFC); ***4**: Reported detection limit 3 CFU/mL; ***5**: Culture first, confirmed by PCR.

single future study. Overall, the *M. bovis* prevalence in IM, independent of individual cow infection status, was estimated at 5% (95%CI: 3%–7%).

In TST positive cows, the summarised prevalence for *M. bovis* in IM stratified by diagnostic test method group is presented in Fig. 3. Slight reductions in the I^2 statistics was observed for culture only studies and combined culture and PCR studies after restricting the analysis to TST positive cows only, however, considerable heterogeneity remained within each diagnostic test method group (I^2 exceeding 83% in both groups). In contrast, the heterogeneity in PCR only studies appeared to increase after restricting the analysis to TST positive cows; little overlap is evident in confidence intervals and $I^2 = 97.23\%$. For each diagnostic test method group, the prevalence estimates for TST positive cows (Fig. 3) appeared slightly higher than the prevalence estimates in cows independent of the individual cow infection status (Fig. 2). Prediction intercals were also wide in TST positive cows highlighting the uncertainty of predicting the value of a single future study. Overall, the *M. bovis* prevalence in IM from TST positive cows was estimated at 8% (95%CI: 4%–13%).

M. tuberculosis was frequently reported in IM samples [5,86,91,94–100], *M. africanum* was also reported in one study [87] (Tables 1–3).

3.2.2. Prevalence of M. bovis in bulk tank milk

Seven studies from four articles were included in the descriptive table for bulk tank milk studies (Table 4). Of these, only Zumarraga et al. (2012) [75] reported the herd infection status from which BTM samples originated.

In the meta-analysis, the *M. bovis* prevalence in BTM, independent of herd infection status, varied considerably depending on the diagnostic test method used, for example prevalence was 0% in one culture only study and 40% in the PCR only study (Fig. 4).

There was little overlap in confidence intervals indicating heterogeneity among culture only BTM studies ($I^2 = 92.6\%$, $p < 0.01$) and there was no evidence of heterogeneity in combined culture and PCR studies ($I^2 = 0.00\%$, $p > 0.99$), however only two studies were included in each sub-group analysis, respectively. Overall, the *M. bovis* prevalence in BTM, independent of herd infection status, was estimated at 5% (95% CI: 0%–21%) (Fig. 4).

Guindi et al. (1980) reported the detection of *M. tuberculosis* in bulk tank milk (Table 4).

4. Discussion

4.1. Prevalence of M. bovis in individual milk and bulk tank milk

This systematic review and meta-analysis was undertaken to estimate the probability of detecting *M. bovis* in raw milk collected on dairy cow farms. In individual milk samples, two estimates were obtained, including 5% (95%CI: 3%–7%) in milk samples collected independent of individual cow TST infection status and 8% (95%CI: 4%–13%) in milk from TST positive cows. In bulk tank milk, independent of herd infection status, the estimate was 5% (95%CI: 0%–21%). These estimates are important, particularly for risk assessment, providing insights into the potential risk of zoonotic tuberculosis transmission from unpasteurised milk and dairy products made using raw milk.

The point estimate for the prevalence of *M. bovis* in IM collected from TST positive cows (Fig. 3) was higher than IM collected independent of individual cow infection status (Fig. 2). This result is expected, as the probability of *M. bovis* shedding in milk will be related to the infection status of the individual cow. Nonetheless, there was considerable heterogeneity in the results from different studies, even after restricting the analysis to TST positive cows alone. This may reflect more advanced infection in settings where surveillance and control strategies for bovine tuberculosis is less rigorous. Animal- and herd-level differences that exist between studies are also likely. Several factors are known to affect the sensitivity and specificity of diagnostic tests for *M. bovis* infection

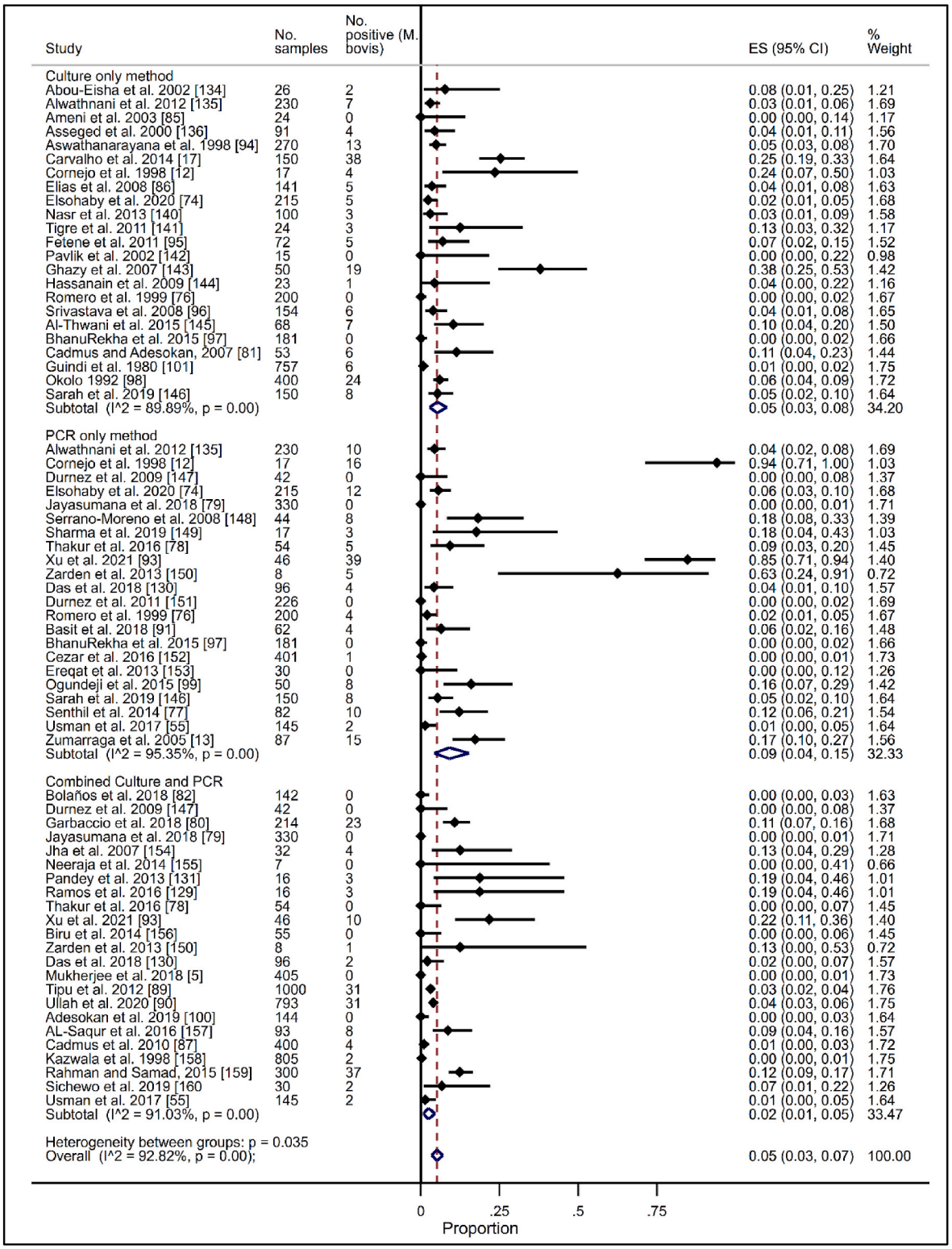

Fig. 2. Meta-analysis with summary estimates, 95% confidence intervals and 95% prediction intervals of the proportion of individual cow milk (IM) samples, independent of individual cow bovine tuberculosis infection status, positive for *Mycobacterium bovis*, stratified by diagnostic test method used to detect *Mycobacterium bovis* bacteria or DNA.

[7]; test sensitivity is particularly influenced by stage of infection and disease, whereas specificity can vary in different locations, depending on the presence of cross-reacting organisms. Persistence of infection in wildlife reservoirs such as in badgers, buffalo or feral pigs can also occur. Genuine variation in the prevalence of infection in different settings is also likely.

As reflected in Figs. 2 and 3, which relate to IM studies, prevalence appeared higher in PCR only studies compared to either culture only studies or combined culture and PCR studies. It is possible that this may reflect a higher analytical sensitivity of PCR compared with culture in

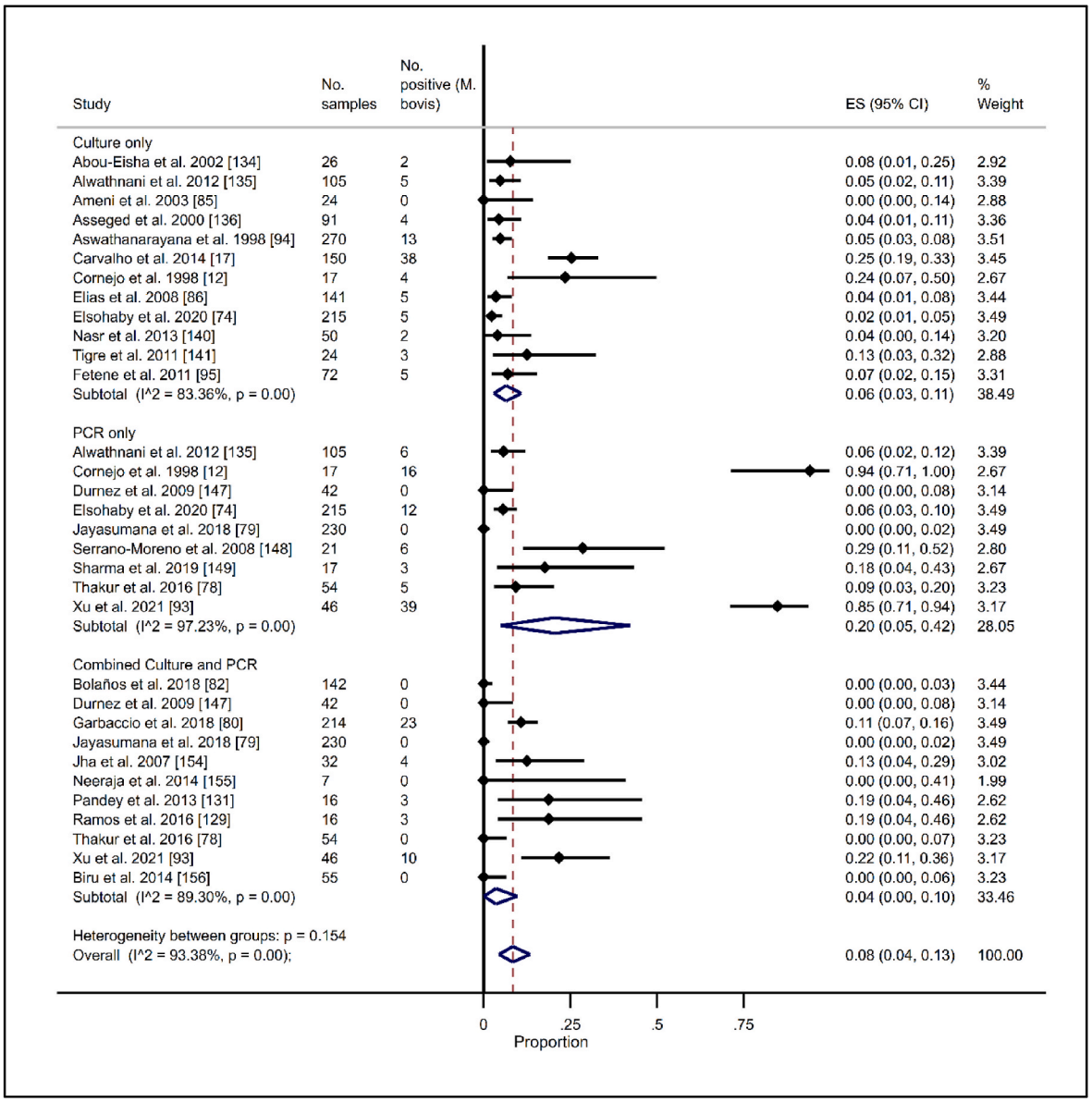

Fig. 3. Meta-analysis with summary estimates, 95% confidence intervals and 95% prediction intervals of the proportion of individual cow milk (IM) samples collected from tuberculin skin test positive cows, positive for *Mycobacterium bovis*, stratified by diagnostic test method used to detect *Mycobacterium bovis* bacteria or DNA.

milk samples. Indeed, some authors have reported a higher analytical sensitivity of PCR on tissue samples compared with culture [102], but not others [103,104]. However, it is not yet clear whether the sensitivity of PCR compared with culture differs in milk samples; further research is warranted. Of note, MTBC DNA can still be present in tissue samples in non-viable bacteria in sufficient quantity to be detected by PCR after purification [105]. This may explain why some authors cited in the present study report higher sensitivity using PCR than culture, because PCR will detect both viable and nonviable bacteria. In contrast, culture methods will detect viable bacteria only. Quantification of the number of viable *M. bovis* bacteria in milk samples would need to be determined to resolve this issue, however, no such study has yet been reported.

It was not possible to quantify the *M. bovis* bacterial load in milk (e.g. the number of CFU/mL) on farm as this was not reported with any of the studies in the current review.

4.2. Methodological considerations

In order to maximize the sensitivity of the search, the search strategy was not limited to prevalence studies. Consequently, many of the included studies did not adequately describe appropriate sample size calculations or choice of sampling schemes used. This may have introduced important sample selection bias. Studies which were designed to measure prevalence to a desired level of precision are more likely to have used appropriate sample sizes and random sample collection methods, hence are more likely to report accurate estimates of *M. bovis* prevalence in milk. In contrast, studies which specifically targeted TST positive cows/herds for milk sample collection are more likely to report higher *M. bovis* prevalence estimates in milk, because *M. bovis* shedding in milk is related to the TST infection status of the animal/herd.

Most studies included in this synthesis originate in non-English speaking countries, with the potential for bias following the exclusion of non-English articles during initial screening. At title and abstract screening, nineteen non-English articles from 13 countries (Argentina,

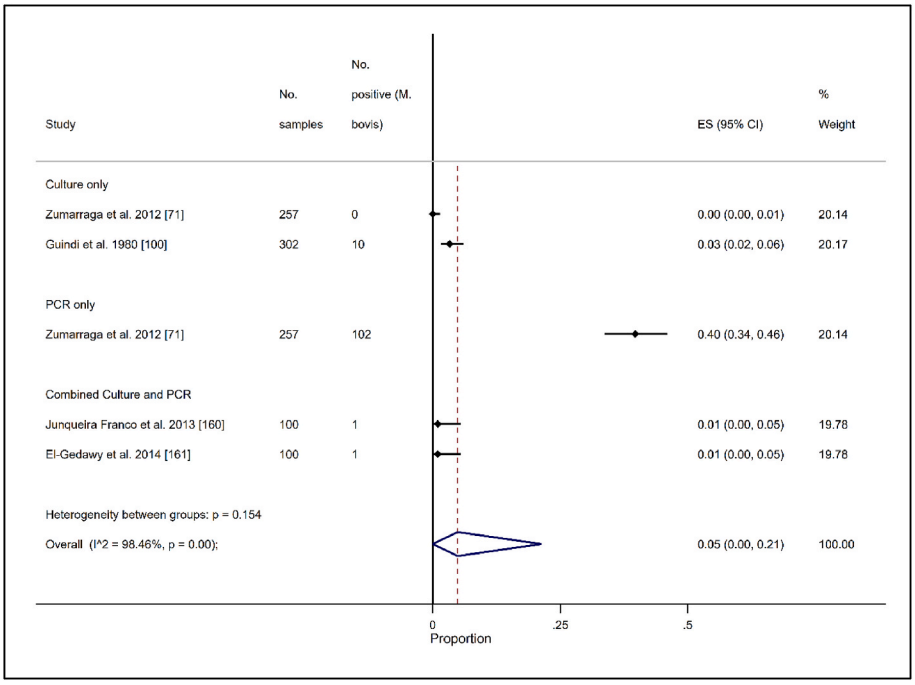

Fig. 4. Meta-analysis with summary estimate and 95% confidence interval of the proportion of bulk tank milk (BTM) samples, positive for *Mycobacterium bovis*, stratified by diagnostic test method used to detect *Mycobacterium bovis* bacteria or DNA. The study by Zumarraga et al. (2012) [75] includes 80 herds considered positive and 177 negative for *M. bovis* infection. The TST infection status of herds in the other three studies (Guindi et al., 1980; Junqueira et al., 2013; El-Gedawy et al., 2014) is not recorded.

Brazil, Bulgaria, China, Côte d'Ivoire, Cuba, Ethiopia, Georgia, Iraq, Mexico, Russia, Taiwan and Turkey) investigating *M. bovis* in milk were excluded [106–124]. Bias was considered minimal or unlikely because six of these countries were represented in the synthesis with other English-language studies. Further, it is possible that some of the 13 excluded non-English articles may not have met the inclusion criteria of this systematic review. This latter possibility could only be determined with an English translation of the full text.

In the current study, publication bias was considered unlikely. With many meta-analyses, there is a focus on significance or magnitude of effect (for example, treatment versus control), which raises concerns that non-significant results or smaller effect estimates are less likely to be published. Here, in contrast, the focus of the present study is on prevalence, where study results (low or high prevalence) would seem less likely to influence a decision to publish. Indeed, it was assumed the most likely reason for not publishing a prevalence study would be the non-detection of *M. bovis* in milk (0% prevalence), however, several of the studies in the meta-analysis reported 0% prevalence in milk. In meta-analyses of studies that aim to measure the proportion with an outcome (rather than to make comparisons), funnel plots have been found to be an inaccurate method of assessing publication bias [125], and were therefore not pursued here. Rather, the issue of heterogeneity in the study results was particularly focused on, and then considering what might explain it.

Heterogeneity was noted in each one of the meta-analyses that were conducted. In broad terms, heterogeneities in a meta-analysis can be clinical or methodological. In the context of the present study, clinical heterogeneity may refer to biological differences in individual cows/herds (e.g. infection status, stage of disease, immune status) whereas methodological heterogeneity may refer to differences in the way that studies were conducted (e.g. study design, sampling strategies, risk of bias).

A heterogeneity indicator such as the I^2 statistic describes the percentage of the variability in prevalence that is due to heterogeneity rather than sampling error, and does not distinguish between the different sources of heterogeneity [126]. Therefore, drawing conclusions from a meta-analysis when clinical heterogeneity is expected in the summary estimate necessitates careful interpretation. When clinical differences are expected, a sub-group analysis may help investigate the clinical heterogeneity [23,127]. For example, in the present study, sub-group analysis in IM by diagnostic test in TST positive cows only indicated that because the heterogeneity became smaller (less heterogeneity was observed) in culture only studies and combined culture and PCR studies, these subgroups may help explain some of the observed heterogeneity. However, it was not possible to explore the observed heterogeneity further due to inconsistency in the quality of reporting across studies and resultant missing information for important variables. For example, there was insufficient detail regarding stage of infection/disease of TST positive cows (noting that cows with advanced disease are more likely to shed bacteria in their milk), and the individual cow/herd TST infection status was often not reported or was unclear.

4.3. Infection status of the individual cow/herd

In the selected studies, the TST infection status of the individual cow or herd was frequently either not reported or was unclear.

Information about the infection status of a herd or animal (at the time of milk sampling) and the infection history of the herd (over the last several years) are of particular importance, given the impact of the stage of infection and disease on the shedding of *M. bovis* in milk. In the current study, efforts to resolve this concern at the IM-level were made by conducting the meta-analysis twice, using all data independent of infection status (Fig. 2) and with the subgroup analysis using data restricted solely to TST positive cows (Fig. 3). However, there are some further challenges relating to the biology of *M. bovis* in cattle to be considered during study interpretation. For example, there is the potential for intermittent shedding of *M. bovis* in the milk of bTB infected cows, which would impact the number of CFU/mL shed in milk from milking to milking. Further, bacterial shedding in milk may also vary with milk yield (as well as by stage of infection and disease, as highlighted above). Some, if not all, of these factors are likely to have contributed to the observed heterogeneity across studies, however, the relative importance of each would be difficult to measure without experimental studies.

For BTM studies, only four articles were identified, with herd TST infection status only reported in one. The meta-analysis was conducted with all available data (Fig. 4) on the assumption that bTB is endemic in each of the countries represented in the BTM studies (Argentina, Brazil,

Egypt; Table 4). In many of the countries represented in the IM studies, extensive livestock systems are common which would not necessitate bulk tanks to store milk; consequently, this may have limited the availability of on-farm bulk tanks to sample from.

4.4. Strength and limitations

A comprehensive search strategy was used across seven scientific databases giving confidence that all the relevant published literature was identified. However, the overall quality of reporting in the selected studies was quite varied. Several studies had to be excluded at full text review because the total number of samples tested, and their corresponding test result, was unclear or not reported. Even the animal species from which BTM samples were collected was not specified in one article [66]. Further, many studies did not report information relating to the diagnostic test used, such as the test sensitivity and specificity, the detection limit of the diagnostic test, or the use of controls.

The studies included in this synthesis principally originate in countries with endemic (generally uncontrolled) bTB in cattle, and few studies were available from counties/regions such as Ireland or the UK where national bTB eradication programs are implemented. It is likely that estimates from countries with national bTB eradication/control programs, if available, would be lower, as all animals are subjected to regular SICTT testing (e.g. at least yearly in Ireland), which should limit the number of TST-positive cows with advanced disease.

Care is needed when interpreting the studies that utilised PCR methods. Among these studies (either PCR only or combined culture and PCR studies), there was considerable variation in the selected PCR target genes and sequences (Tables 2 and 3), and lack of consistency among primers used for these targets. The use of molecular methods such as PCR for the detection of *M. bovis* can be problematic as members of the MTBC share 99.9% similarity at the nucleotide level [10]. Investigating the presence or the absence of the mycobacterial regions of difference (RD) 4 and RD9 is considered important to differentiate *M. bovis* and animal-adapted tubercle bacilli from other members of the MTBC, in particular the RD4 locus which is deleted from *M. bovis* [128]; however only a small number of studies investigated RD4 and/or RD9 [71,79,83,87,88,93,100,129–131]. It is therefore plausible that some of the positive results in PCR only studies or combined culture and PCR studies that targeted genes/sequences other than RD4 and RD9, may be due to other members of the MTBC, rather than *M. bovis* specifically. Indeed, several authors reported the detection of *M. tuberculosis* in milk [5,82,93–100]. Further, *M. orygis* is emerging as an important risk in terms of zoonotic tuberculosis in South Asia [132,133] and warrants further investigation. This variation in MTBC zoonotic agents may also help explain some of the observed heterogeneity in studies using PCR to detect *M. bovis* in particular. In contrast, there was less variation in the culture methodologies described in culture only studies (Table 1), consistent with less evidence of heterogeneity observed in these studies.

4.5. Study implications

To the author's knowledge, this is the first study to synthesise the relevant international literature investigating the probability of detecting *M. bovis* in on-farm milk in this level of detail. These estimates can be used to help inform risk assessments (qualitative and quantitative) relating to the potential risk of zoonotic tuberculosis from unpasteurised milk and dairy products made using raw milk. The outputs of these risk assessments can help inform policy decisions relating to the prevention and control of zoonotic tuberculosis.

5. Conclusion and recommendations

The reported prevalence and 95% C.I. of *M. bovis* in individual cow's milk was estimated to be 5% (95%CI: 3%–7%) (in cows independent of TST infection status) and 8% (95%CI: 4%–13%) (in TST positive cows), and 5% (95%CI: 0%–21%) (in herds independent of TST infection status) in bulk-tank milk. The *M. bovis* prevalence in TST positive cows appeared greater than the prevalence among all cows independent of TST infection status. However, these estimates need to be interpreted and generalised with caution. Considerable heterogeneity was observed among studies, while variation in the quality of reporting was also identified, including missing information that prevented further investigation of the observed heterogeneity. Further, these estimates are principally derived from countries with endemic (generally uncontrolled) bovine tuberculosis. Nonetheless, this is the first study to synthesise the relevant international literature investigating the probability of detecting *M. bovis* in on-farm milk in this level of detail. This study highlights the risk of zoonotic transmission of *M. bovis* via unpasteurised milk and dairy products made using raw milk.

Ethical approval

Ethical approval was not required.

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The lead investigator is a full-time employee of The Irish Department of Agriculture, Food and the Marine (DAFM).

Author contributions

Conceptualization, Á.C and S.M.; Methodology, Á.C. and S.F.; Searches and data collation, Á.C.; Record screening, Á.C.; Data extraction and Curation, Á.C.; Formal analysis, Á.C.; Writing—Original Draft Preparation, Á.C.; Writing—Review & Editing, Á.C., S.G., S.F., and S.M.; Supervision, S.F. and S.M. All authors read and approved the final manuscript.

Declaration of competing interest

All authors have a specialist interest in One Health and zoonotic diseases. All authors declare no conflicts of interest.

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Supplementary file

Search strategies used in database searches

PubMed (n = 400 records retrieved)

((((((*Mycobacterium bovis*"[Title/Abstract]) OR ("M. bovis"[Title/Abstract])) OR ("m.bovis"[Title/Abstract])) OR ("bovine tuberculosis"[Title/Abstract])) OR ("btb"[Title/Abstract])) OR ("bovine tb"[Title/Abstract])) AND (milk[Title/Abstract])

Web of science (n = 535 records retrieved)

TS=("bovine tb") OR TS=("btb") OR TS=("m.bovis") OR TS=("M. bovis") OR TS=("bovine tuberculosis") OR TS=("*Mycobacterium bovis*") AND TS=("milk")

CAB Abstracts (n = 856 records retrieved)

(("bovine tb") OR ("*Mycobacterium bovis*") OR ("bovine tuberculosis") OR ("M. bovis") OR ("m.bovis") OR ("btb") AND ("milk")) AND (((sc:(("CA")))))

Science direct (n = 128 records retrieved)

("*Mycobacterium bovis*" OR "bovine tuberculosis" OR "btb" OR "bovine tb" OR "M. bovis" OR "m.bovis") AND ("milk")

Embase ("Embase Classic+Embase 1947 to 2021 July 01") (ALL FIELDS) (n = 639 records retrieved)

(("*Mycobacterium bovis*" or "bovine tuberculosis" or "bovine tb" or "M.

bovis" or "m.bovis" or "btb") and "milk").af.

Medline ("Ovid MEDLINE(R) ALL 1946 to July 01, 2021") (ALL FIELDS) (n = 466 records retrieved)

(("Mycobacterium bovis" or "bovine tuberculosis" or "bovine tb" or "M. bovis" or "m.bovis" or "btb") and "milk").af.

Scopus (n = 666 records retrieved)

(TITLE-ABS-KEY ("*Mycobacterium bovis*") OR TITLE-ABS-KEY ("bovine tuberculosis") OR TITLE-ABS-KEY (btb) OR TITLE-ABS-KEY ("bovine tb") OR TITLE-ABS-KEY ("M. bovis") OR TITLE-ABS-KEY (m. bovis) AND TITLE-ABS-KEY (milk))

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