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Review article

What counts? A review of *in vitro* methods for the enumeration of *Mycobacterium avium* subsp. *paratuberculosis*

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ABSTRACT

Enumeration of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from clinical samples or from inoculum suspensions for challenge studies is an essential pre-requisite for paratuberculosis research. However, MAP organisms are slow and challenging to grow *in vitro*, with a strong tendency to clump. Standard bacteriological methods are not well suited to enumerate MAP. Enumeration can be achieved by culture-based methods, direct microscopic counts, turbidimetry, pelleted weights and quantitative PCR. There is an unresolvable discrepancy between culture-based methods, enumerating cultivable MAP organisms, and other methods enumerating total MAP organisms irrespective of viability, hence these methods are not directly comparable. However, they are complementary and should be used in parallel when accuracy is required. We review currently available methods, their principles, limitations and equivalence between methods.

1. Introduction

Historically, quantification of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) for experimental studies in animals has focused on inoculum dose, mainly reported as wet or dry weight of pure bacterial culture (Karpinski and Zorawski, 1975; Kluge et al., 1968). The first report of enumeration of viable MAP in a sample was by Brotherston et al. (1961). Nowadays a variety of enumeration methods are available, each with inherent technical characteristics and sources of bias.

The ability to detect and enumerate MAP is fundamental to interpretation and comparison of results of epidemiological studies of paratuberculosis (PTB) (Reddacliff et al., 2003b). Taking account of biases associated with enumeration of MAP in vitro also contributes to standardizing research using animal models of PTB (Begg and Whittington, 2008). The exposure dose of MAP has a profound impact on the outcome of infection (Marquetoux et al., 2018). Interpretation of this effect from experimental challenge studies is hampered by the heterogeneity of reported enumeration techniques.

This paper reviews currently available *in vitro* MAP enumeration methods. It aims to clarify similarities and differences between methods. This in turn assists interpretation and comparison of experimental studies involving MAP challenge using different enumeration methods. Since enumeration of MAP, especially in samples from clinically affected animals, relies on the accuracy of the method to detect organisms in the first place, the sensitivity of the method is therefore considered in parallel with MAP enumeration *per se*.

2. Methods for MAP enumeration

2.1. Culture-based methods

Culture-based enumeration methods are the most commonly reported. They are used to determine the number of viable MAP in a clinical sample or in a suspension obtained after culture. Culture is based on the ability of MAP to grow *in vitro*, therefore only cells actually forming a colony are enumerated following culture. Non-viable, and under some circumstances viable non-growing MAP, will not be enumerated.

Irrespective of the technique, MAP organisms have long been known to be difficult to grow *in vitro* (de Juan et al., 2006; Stevenson, 2015). Organisms isolated primarily from cattle (type II strains) were more readily cultured than sheep strains (type I strain), with a more satisfactory isolation rate from cattle clinical samples (Whittington and Sergeant, 2001). Differential growth of cattle versus sheep strains was also reported by Fernandez et al. (2014). Plate counts underestimated the number of sheep strains of MAP by two $\log_{10}$ units, unlike cattle

Abbreviations: AFB, acid fast bacilli; CFU, colony forming units; Ct, cycle threshold; MAP, *Mycobacterium avium* subsp. *paratuberculosis*; MPN, most probable number; OD, optical density; PCR, polymerase chain reaction; PTB, paratuberculosis; ZN, Ziehl-Neelsen

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strains. Additionally, liquid broths outperform solid media for the growth of MAP, in particular for sheep strains (Whittington et al., 2013, 1999). Lack of sensitivity of solid media for sheep strains means that MAP isolation from sheep samples, or from cattle cross-infected with strain type I, could be biased towards the detection of fast growing MAP types (Whittington and Sergeant, 2001). A differential bias in enumeration could thus occur, whereby plate colony counts could grossly underestimate the inoculum dose prepared with sheep strains. Further, differential bias can occur due to greater tendency of the cattle strain to clump in suspension, as addressed below.

2.1.1. Solid media culture

This method is widely used to enumerate the inoculum dose in pathogenesis and epidemiological studies (Begg and Griffin, 2005; Brotherston et al., 1961; Stewart et al., 2004). It gives an estimation of the number of colony-forming units (CFU) growing on slopes or plates, from serial dilutions of an inoculum.

Culture on solid media often requires prolonged incubation periods (de Juan et al., 2006). Detectable growth commonly occurs in 4–6 weeks for cattle strains, but can be up to 52 weeks for sheep strains (de Juan et al., 2006; Stevenson, 2015). Colonies can be difficult to detect visually and to count, hence interpretation of plate counts can be subjective (Elguezabal et al., 2011). Results tend to be poorly reproducible and highly variable (Elguezabal et al., 2011; Kralik et al., 2012; Reddacliff, 2002). Inherent lack of sensitivity of solid media culture, particularly for the isolation of “slow growing” strains, limits its analytical sensitivity. Therefore it is recommended not to use solid media as a routine enumeration technique for MAP of sheep origin, especially for culture of clinical specimen (Reddacliff, 2002). However, solid media culture is still widely used in sheep PTB pathogenesis studies to enumerate viable MAP both in pure culture and tissue homogenate (Delgado et al., 2013). The choice of an appropriate medium and long enough incubation times can improve isolation and culture of MAP on plates. Discrepancies in detection times of up to 10 weeks were observed between different solid media for the same isolates (Whittington et al., 2011). Optimal and reliable growth for a range of MAP strains and hosts was obtained with modified Middlebrook 7H10 agar.

2.1.2. Liquid media culture

Liquid media are sensitive substrates, detecting slow growing strains more readily than solid media. Their use improves detection rate in samples from animals with clinical Johne’s disease (Motiwala et al., 2005; Whittington et al., 1999), increases accuracy of viable MAP number estimates, and is faster than solid media culture (Damato et al., 1983; Damato and Collins, 1990). Estimates are based on endpoint titration or time to growth in liquid medium.

2.1.2.1. End point titration. This method, also known as the most probable number (MPN) method, employs serial dilutions of MAP suspensions in liquid medium until no growth is observed. This technique is more accurate than direct colony count on solid media, but it is expensive and time consuming to confirm the absence of growth at high dilutions (Reddacliff et al., 2003a). Accuracy is also affected by clumps, especially towards the end point (Reddacliff, 2002). This method was used in at least one sheep infection model to enumerate colony forming units in inoculum doses (Reddacliff and Whittington, 2003), and in another study to calculate the excretion of viable MAP in infected sheep faeces (Whittington et al., 2000).

2.1.2.2. Time to growth in liquid media

2.1.2.2.1. Bactec. An automated radiometric detection system for bacterial growth in liquid medium was developed using the Bactec instrument (Bactec 460, Johnston Laboratories, Towson, Md.) to analyse the amount of radioactive CO_2 released by bacterial metabolism (Reddacliff, 2002). This method was based on modified 12B radiometric medium (BACTEC 12B; Becton Dickinson, Sparks, Md.) (Shin et al., 2007). Detection of MAP within 10 days was achieved using this system (Damato and Collins, 1990), which displayed better sensitivity than other culture methods for sheep strains (Whittington et al., 1999).

Regression analysis can be used to establish equations relating the time to reach a threshold growth index, rather than an entire growth curve, and the inoculum size (Reddacliff et al., 2003a). This method was used for field isolates of sheep strains from faeces or tissues (Reddacliff et al., 2003a). Using the same equations for routine enumeration in subsequent samples infected with the same isolate, the precision was of one to two $\log_{10}$ units, improving for higher concentrations (Reddacliff et al., 2003a). This method has shorter turnover time and lower cost than other methods, to achieve similar precision. It is also less affected by clumps in the broth (Lambrecht et al., 1988; Reddacliff et al., 2003a). The Bactec system was extensively used in ovine PTB studies as an alternative to plate counts, but the Bactec 12B medium was discontinued in 2012 (Whittington et al., 2013).

2.1.2.2.2. MGIT ParaTB. For cattle isolates, a non-radiometric liquid medium, MGIT ParaTB (Becton Dickinson, Sparks, MD), has been used successfully as an alternative to the radiometric Bactec. It automatically or manually detects bacterial growth by fluorescence (Shin et al., 2007). It achieves early detection and has high sensitivity. For enumeration, standard growth curves of number of CFU in the inoculum versus time to detection were validated for bovine strains (Shin et al., 2007), producing precise estimates, with prediction error below one $\log_{10}$ unit, closely matching those obtained with radiometric Bactec. For the detection and enumeration of sheep strains, an alternative modified Middlebrook 7H9 broth was specifically developed and validated. This method is not automated and relies on end point titration (Whittington et al., 2013).

2.1.3. Sources of variation of viable cell counts using culture methods

Any condition affecting the viability of MAP cells directly affects the accuracy of culture-based enumeration methods. Here we consider storage, decontamination, dormancy and clumping.

2.1.3.1. Storage. Raizman and Espejo (2011) used culture techniques to study MAP survival in stored faecal samples from naturally infected cows. The odds of observing a decrease in MAP load from high to low or low to negative was 1.13 per month. The loss of viability in samples stored at −18 °C compared with −70 °C was not significant. The number of thawing/refreezing cycles had no effect on survival time. Sheep strains showed a minimal loss of viability when stored at −80 °C for over a year, but a decrease of one to two $\log_{10}$ units was observed in the first three months during one year of storage at −20 °C (Reddacliff, 2002).

2.1.3.2. Decontamination. Decontamination is critical for MAP culture from clinical samples, particularly in liquid cultures, but can impact upon MAP viability. Routine decontamination protocols reduced MAP numbers of three $\log_{10}$ units in sheep faeces or tissues, thus reducing the sensitivity of detection for samples with a low infection rate (Reddacliff et al., 2003b). The lower detection limit was 250 MAP/g in faeces in liquid culture medium, and at best 80 MAP/g in tissues when using centrifugation.

2.1.3.3. Dormancy. A dormant state exists during which MAP organisms are viable but non-cultivable (Whittington et al., 2004). This can affect culture-based enumeration methods, especially in late stages of growth, as nutrient depletion in the culture medium might favour MAP dormancy (Elguezabal et al., 2011). The presence of non-cultivable MAP would have the effect of increasing growth time, thus underestimating the number of viable MAP.

2.1.3.4. Clumping. Most enumeration methods, and in particular those based on culture, are biased by the strong tendency of MAP cells to

clump both in vitro and in vivo and to form aggregates with cell debris or broth medium (Kralik et al., 2012; Reddacliff, 2002). Hence one CFU in culture may in fact correspond to a clump of several cells, sometimes in large numbers, rather than a clone from one unique MAP cell. The extent of clumping is variable. Brotherston et al. (1961) mention the presence of single bacilli or small clumps with rarely more than 10 bacilli per clump in a suspension of cattle strain of MAP. By contrast, Klijn et al. (2001) evaluate the underestimation factor due to clumping at two to three $\log_{10}$ units.

Thorough dispersion of MAP cells in suspension is a prerequisite to avoid underestimation of cell numbers due to clumping. Treatment options include the use of Tween-20 or Tween-80 acting as a dispersant (Hughes et al., 2001; Reddacliff, 2002), repeated passages through a fine gauge needle (Begg et al., 2010; Reddacliff et al., 2003a), filtering through a micro-pore (Lambrecht et al., 1988), vortexing (Elguezabal et al., 2011), bead-beating with silica beads (Kralik et al., 2012), continuous stirring of the broth (Hughes et al., 2001), constant shaking at 300 rpm during incubation (Peñuelas-Urquides et al., 2013), and sonication (Stabel et al., 1997). Time to growth in liquid medium is less affected by clumping compared to other culture-based methods (Reddacliff et al., 2003a).

Clumping in suspensions can be assessed by direct light microscopic examination (wet mount), and has been performed in many paratuberculosis studies. If clumping cannot be totally avoided, other lab procedures can help mitigate the problem as described by Hughes et al. (2001) and Lambrecht et al. (1988) who reported achievement of single bacilli suspensions. However, others highlight difficulties in overcoming clumping despite preventive procedures (Kralik et al., 2012). There is evidence that sheep strains are readily emulsified in suspension, with only occasional clumps of two or three organisms, while cattle strain tended to form many large clumps of bacilli despite the use of procedures to limit clumping (Reddacliff, 2002).

2.2. Direct microscopic counts

Microscopic counts of MAP, either in a bacterial suspension or stained smears, are frequently reported. Direct counts are faster than culture-based methods, but direct counting estimates the total number of bacteria including viable and non-viable or non-cultivable organisms.

2.2.1. Using counting chambers

This method involves spreading a known volume of unstained MAP suspension on a slide equipped with a counting chamber or haemocytometer. A grid enables counting of cells in representative microscopic fields and extrapolation to estimate MAP concentration.

The technique requires that the number of cells is large enough to allow quantification but without overlapping, thus determining the appropriate dilution factor can be time consuming (Elguezabal et al., 2011). The presence of large clumps, media remnants, cell debris or other material can limit reliability or even meaningful results (Dennis et al., 2010; Elguezabal et al., 2011). This technique can thus only be used on pure MAP cultures. In clinical samples, a direct count on stained smears is preferable. Like all techniques based on MAP physical properties, it does not distinguish between viable and non-viable MAP. Hence, the number of infectious cells is likely overestimated.

Direct count in a counting chamber is less frequently reported than other enumeration techniques and is usually performed in parallel with other techniques (Begg et al., 2010; Hughes et al., 2001; Reddacliff and Whittington, 2003).

2.2.2. Using stained smears

The most common stain for Acid Fast Bacilli (AFB) in veterinary medicine is Ziehl-Neelsen (ZN) colouration (Begg et al., 2010; Stewart et al., 2004; Verna et al., 2007). Haematoxylin-eosin (Delgado et al., 2013; Whittington et al., 1999), ZN plus haematoxylin-eosin (Begg et al., 2010; Delgado et al., 2013; Pérez et al., 1996), the Kinyuon method (Gwozdz et al., 2000), and auramine staining (Damato and Collins, 1990) are also reported.

Stained smears have been reported to assess faecal shedding in infected animals (Stewart et al., 2004; Whittington et al., 2000). However, MAP organisms are difficult to distinguish from other AFB, causing a lack of specificity in the presence of other AFB. Merkal et al. (1968) reported that 76% of cattle were AFB positive in faecal smears in the absence of confirmatory evidence of infection with MAP. Faecal smears also lack sensitivity, with reports of negative faecal smears from sheep in the multibacillary stage of PTB (Whittington et al., 2000). These observations preclude this method for accurate diagnosis of faecal shedding, or estimation of shedding levels.

Stained smears are also used to detect AFB in clinical tissue samples, alongside histopathology. MAP organisms are smaller and less rod shaped than other *Mycobacteria* (Jeyanathan et al., 2007). Accurate detection of MAP using light microscopy is thus more difficult than that of Mycobacterium tuberculosis, requiring oil-immersion microscopy with 1000x magnification (Delgado et al., 2009; Jeyanathan et al., 2007), with a similar limit of detection of approximately 10^4 AFB/mL (Jeyanathan et al., 2006). The relative frequency of paucibacillary and abacillary lesions in PTB is a limitation to the use of stained smears. Moreover, clumping, especially by the cattle strain, can make enumeration with this technique impossible (Begg et al., 2005).

To conclude, stained smears are routinely used for detection of MAP in clinical samples. In experimental infection models, they are reported to estimate MAP doses in inocula prepared directly from tissue homogenates (Begg et al., 2010; Gwozdz et al., 2000; Stewart et al., 2004; Verna et al., 2007). Due to important limitations, they should be used preferentially as a confirmatory test of culture-based methods.

2.3. Methods based on physical properties of MAP

These methods estimate the total number of MAP cells irrespective of viability.

2.3.1. Turbidimetry

This method is based on light absorbance, using the relationship between the turbidity of a liquid suspension and cell concentration. It is a relatively quick and simple technique. Absorbance can be related to concentration using a nephelometer or a spectrophotometer. Either method can only be used with liquid culture. The use of a medium containing egg yolk is precluded with this technique, potentially requiring subculture in a different medium, typically Middlebrook 7H9 broth supplemented with Middlebrook OADC (oleic acid, albumin, dextrose, catalase) (Kralik et al., 2012; Peñuelas-Urquides et al., 2013). Alternatively, bacterial pellets obtained after centrifugation can be washed and re-suspended in phosphate buffered saline (Elguezabal et al., 2011; Fernandez et al., 2014).

Measures of turbidity are expressed as optical density units (OD) or at wavelength between 550 and 600 nm. Several equations were calibrated to relate OD readings and MAP cell concentration (Kralik et al., 2012). Turbidity can also be expressed in McFarland units (Fernandez et al., 2014; Kralik et al., 2012; Verna et al., 2007), or in Brown's scale as reported in earlier studies (Brotherston et al., 1961). The McFarland scale is read by visually comparing the suspension with a physical standard in a tube, based on *E. coli* organisms. However, physical properties of *E. coli* differ from those of *Mycobacteria* sp. Hence some authors recommend that McFarland units should not be used to enumerate MAP numbers directly (Peñuelas-Urquides et al., 2013). Instead, they need to be calibrated specifically for MAP, similar to OD readings, by comparing number of viable MAP with plate counts (Kralik et al., 2012) or direct visual counts (Hughes et al., 2001). One study showed excellent fit between number of MAP estimated by qPCR and the McFarland scale (Kralik et al., 2012). This suggests that the McFarland scale does provide better estimates than MAP-specific calibration based

on culture. However, turbidimetry does not distinguish between live or dead cells, hence McFarland units do not necessarily reflect the growing potential of MAP in the sample or inoculum (Peñuelas-Urquides et al., 2013).

Turbidimetry performs better in concentrated suspensions, and has a detection limit of 10^7 cells/mL (Elguezabal et al., 2011; Reddacliff, 2002). The precision of the relationship between number of cells and turbidity is likely affected by the presence of clumps (Peñuelas-Urquides et al., 2013), which could contribute to poor repeatability between trials (Reddacliff, 2002).

Turbidity methods are useful to obtain standard MAP suspensions of approximately equivalent MAP concentrations. Where precise estimates of viable MAP numbers are needed, turbidity methods are often followed by another culture-based method, giving a retrospective estimate of the dose of viable MAP (Brotherston et al., 1961; Fernandez et al., 2014).

2.3.2. Pelleted weight

This consists of measuring the weight of a pellet after centrifugation of a MAP suspension. The pellet is usually obtained from a liquid culture medium but can use re-suspended colonies harvested from a plate. The pellet can be measured as wet weight, after simple draining (Hines et al., 2007), dry weight after desiccation (Kluge et al., 1968), or semi-dried weight (Karpinski and Zorawski, 1975).

Weight of a pure culture can be related to the number of viable bacilli using other enumeration techniques, or by a theoretical calculation based on physical properties of MAP, as discussed below. The pellet weight method can be biased by different sizes of MAP bacilli or differences in the water content of different strains (Elguezabal et al., 2011). It is only suited for concentrations over 10^6 MAP/mL, to minimise the error due to extra water content which is approximately two $\log_{10}$ units for suspensions around 10^5 MAP/mL (Elguezabal et al., 2011). Advantages are that the weight of pelleted bacteria is not affected by clumping or loss of growing ability and it is a fast and easy way to visualise MAP quantity. As for turbidity estimates, the conversion between pelleted weight and actual MAP numbers requires using published equivalence figures. Alternatively, a viable count by culture can be done in parallel, for retrospective validation.

Standard procedures has been recommended to obtain bacterial pellets (Hines et al., 2007). Associated with retrospective CFU counts by serial plate dilution, wet weight enumeration has been recommended as the reference method for reproducible enumeration in experimental infection models (Hines et al., 2007).

2.4. Direct quantitative PCR

Quantitative PCR was recently developed to enumerate the genomic equivalent of MAP organisms. The specificity of IS900-based PCR is excellent for MAP detection, with no false-positive detection in dedicated studies (Möbius et al., 2008; Plain et al., 2014).

The number of amplification cycles (Ct) required to reach a threshold level of fluorescence is inversely proportional to the quantity of targeted DNA templates in the PCR reaction and is correlated with the number of organisms or genome equivalents. Quantification requires calibration of each PCR run by including dilutions of external control standards of the target DNA at known concentration. The relative quantity of target DNA in the sample is estimated by interpolating the Ct value with that of the standard curve (Fang et al., 2002), a process termed absolute quantification. Results are expressed either as number of target copies or as picograms of DNA in the template. Plasmids inserted with a copy of the target sequence or genomic DNA can be used as standards (Fang et al., 2002; Sevilla et al., 2014). However, genomic DNA should be preferred for robust enumeration (Yun et al., 2006).

IS900 is often used for quantitative PCR, as this multiple copy sequence is a reliable target for both detection and enumeration (Plain et al., 2014). However, for enumeration, some authors prefer single-copy sequences for the PCR probe, typically f57 (Elguezabal et al., 2011; Kralik et al., 2010; Sevilla et al., 2014). The number of copies of IS900 in MAP genome varies with the strain, reportedly from 15 to 20 (Green et al., 1989) or 14 to 18 (Bull et al., 2000). As a result, if the number of copies differs from that in the standard, enumeration could be affected, although the extent of the error is low, necessarily less than (20-14)/14 = 43%. This is negligible compared with other sources of variation, such as the presence of non-viable MAP, which can affect counts by several orders of magnitude.

While quantitative PCR is not affected by clumping or the presence of non-cultivable MAP in the sample, it typically does not distinguish between viable and non-viable MAP. However, a recent study, combining F57 qPCR and propidium monoazide treatment, showed the possibility to distinguish live and dead MAP cells using qPCR, although the technology required further optimisation (Kralik et al., 2010).

At lower concentrations, as typical in clinical samples, underestimation of numbers could also occur due to larger proportional DNA loss during the extraction process (Elguezabal et al., 2011). Automated purification methods using magnetic beads are deemed to display higher analytical sensitivity than standard spin-column methods, with the added advantage of higher throughput and mitigating the risk of cross-contamination (Plain et al., 2014). With this technique, the analytical sensitivity of quantitative PCR is 10 MAP/gram of faecal sample (Plain et al., 2014; Sevilla et al., 2014). Results obtained by quantitative PCR are more precise and more reproducible than those obtained by plate counts (Elguezabal et al., 2011).

Direct quantitative PCR is not subject to biases described for other methods, in particular clumping. This method thus represents a sensitive and accurate alternative to detect and enumerate total MAP cells, both in clinical samples or MAP inocula.

3. Comparison of estimates between methods

For culture-based methods, slow growth, the presence of non-cultivable MAP and clumping can produce gross underestimates of viable cell number. On the other hand, methods not based on culture cannot distinguish between viable and non-viable MAP, thus overestimating the number of infectious units (Hines et al., 2007). Among methods presented here, culture-based methods, light microscopy and turbidimetry can be affected by clumping, while pelleted weight and direct PCR are not. A summary of findings is available in Table 1.

3.1. Direct microscopic counts using a counting chamber versus end point titration

Reddacliff (2002) compared results from counting chambers with endpoint titration in a series of trials. Mostly, counts using a Thoma counting chamber were significantly higher than those obtained by endpoint titration. In some trials, numbers of viable MAP were similar to total number estimates whereas in others viable MAP counts were up to two $\log_{10}$ units lower than total counts. When suspensions were treated to reduce clumping, differences between direct counts and MPN estimates decreased to less than half a $\log_{10}$ unit. This suggests that the apparent difference between a viable count and a total number of MAP is partially due to an underestimation of the number of viable MAP by culture methods because of organism clumping. However, in samples containing both viable and non-viable MAP, direct counts can also overestimate the number of viable organisms by up to one order of magnitude (Reddacliff and Whittington, 2003). A difference of one $\log_{10}$ unit means that up to 90% of the bacteria present in suspension might be non-viable.

3.2. Direct microscopic counts using a counting chamber versus plate count

For sheep strains of MAP, the number of organisms in both pure culture or tissue samples was determined by microscopic counting, and

Table 1
Summary of equivalence between enumeration methods, based on literature reviewed in this paragraph. End point titration and plate counts (in bold) represent counts of viable/cultivable MAP, all others represent total MAP numbers.

	End point titration	Plate count (CFU)	Total MAP[a]	Direct microscopic count	qPCR (number of genome equivalents)
End point titration				similar to 2 log units lower[d]	
Plate count (CFU)	0.5 to 2 log unit lower[b]			1 to 2 log units lower [b,d]	2 log units lower[b,d]
1 mg wet weight		10^8 to 10^9	$7.5*10^8$		$3.4*10^7$
1 mg dry weight		10^9 to 10^{10}[c]	$5*10^9$		
1 McFarland unit		10^6 to $5*10^7$[b]			10^8

[a] theoretical calculation based on MAP physical properties.
[b] underestimation of viable count by plate count is greater for sheep strains than for cattle strains. In particular, plate counts should not be used for sheep strain isolated directly from a tissue homogenate.
[c] figures corrected to account for clumping, cattle strain of MAP.
[d] underestimation of viable count by culture methods decreases with the use of de-clumping methods.

confirmed retrospectively by plate counts by Begg et al. (2005), with similar results. In another study, MAP concentration in low and high inoculum doses was estimated by direct microscopic counts as 1.2×10^3 MAP/mL and 3×10^8 MAP/mL, respectively but when evaluated using culture on Lowenstein-Jensen and 7H11 media there was 10^2 CFU and 4×10^6, respectively (Delgado et al., 2013). Plate counts thus underestimated MAP numbers by a factor of 10–100. Similar results were obtained for cattle strains, with estimates by plate counting one to two $\log_{10}$ units lower than with direct microscopic count (Sevilla et al., 2014).

3.3. Direct microscopic counts on ZN stained smears versus end-point titration

Direct counts on ZN-stained smears and MPN were carried out in parallel on a filtrated suspension from homogenate tissue samples of sheep (Begg et al., 2010). The number of viable MAP estimated by end-point titration was 2.3×10^8 /mL while that by direct microscopic count was 3.7×10^8 AFB/mL. The suspension was treated before enumeration to maximise MAP recovery and minimise clumping with results suggesting that at least 60% of the MAP organisms were viable.

3.4. Plate count versus end-point titration

In a series of trials, colony counts on plates (modified Middlebrook 7H10 agar) consistently underestimated the number of viable MAP compared to end-point titration (Reddacliff, 2002). For suspensions obtained from pure culture of various sheep strains and one cattle strain, colony counts were lower but the difference with MPN was limited to half to one $\log_{10}$ unit and not significant in most trials. For the cattle strain suspension treated to limit clumping, the difference was less than half a $\log_{10}$ unit, which was not significant. With some sheep strain cultures however, large variation of plate colony counts between replicates was observed, and the magnitude of the difference with MPN was up to two $\log_{10}$ units. For sheep strains obtained directly from tissue homogenates, colony counts gave inconsistent results. Absence of colonies was noted even for samples containing approximately 10^8 to 10^9 viable MAP as per end-point titration. This highlights potential limitations of enumerating viable MAP on solid media, more specifically for clinical specimens of sheep. Begg and Wittington (2008) reported that colony counts typically underestimate the viable count by several orders of magnitude compared to counts by end point titration in liquid media.

3.5. Weight of pelleted bacteria versus "theoretical" total number or other methods

Brotherston et al. (1961) originally attempted to enumerate viable MAP colony counts on solid media. They limited the clumping effect, reporting rarely more than 10 bacilli per clump. They determined that 2 mg wet weight of pure MAP culture was equivalent to approximately 10^8 to 10^9 CFU by plate serial dilution.

Retrospective verification of those estimates was attempted by Reddacliff (2002), based on MAP physical properties. Considering an average size of MAP at 1 µm long and specific gravity similar to *Mycobacterium tuberculosis*, a theoretical number of 5×10^9 MAP/mg dry weight was calculated. Assuming 85% water content for MAP organisms, this corresponds with a theoretical number of 7.5×10^8 MAP/mg wet weight. Accounting for clumping with a 5-fold underestimation correction, estimates in Brotherston et al. (1961) corresponded to 1.6×10^9 to 1.6×10^{10} MAP/mg dry weight. These figures show good agreement with theoretical numbers based on physical characteristics of MAP cells (Reddacliff, 2002). This indicates that CFU counts in Brotherston et al. (1961) were relatively accurate and that bias due to clumping probably resulted in no more than a 5-fold underestimate. However, it should be noted that the length of MAP organism may vary between one and two micrometres (Elguezabal et al., 2011), which may influence weight.

Kluge et al. (1968) provided similar estimates with 1 mg dry weight of MAP suspension containing approximately 10^8 organisms as per plate counts. Other estimates were lower.

Elguezabal et al. (2011) found 1 mg wet weight of MAP equivalent to 3.75×10^6 CFU, on average for eight strains at different time points of the growth curve. That study also concluded that viable counts obtained by plating grossly underestimates the number of MAP, although the same experiment showed that 1 mg wet weight was also equivalent to only 3.39×10^7 MAP genome equivalent estimated by qPCR, which is less subject to underestimation than colony counting. It was not possible to establish distinct patterns for type I and II strains, hence results were reported overall (Elguezabal et al., 2011). Juste et al. (1994) reported that 150 mg wet weight represented 1.36×10^6 CFU of cattle isolate. This represents less than 10^4 MAP/mg wet weight. This figure seems to be strongly underestimated, possibly due to excessive clumping.

In guidelines for experimental infection models of PTB (Hines et al., 2007), they suggest that 1 mg pelleted wet weight is approximately equivalent to 1×10^7 CFU, although no experimental evidence was provided.

3.6. McFarland units versus qPCR or direct microscopic count

Using eight isolates including sheep and cattle strains, Elguezabal et al. (2011) found that one McFarland unit was equivalent to 1.2×10^8 genome equivalent by quantitative PCR. This is very close to the theoretical number of expected cells according to the McFarland standard. It is also in line with other results (Kralik et al., 2012), confirming an excellent fit between MAP numbers determined by qPCR and theoretical *E. coli* numbers according to the McFarland standard. The result is valid for cultures of MAP at different concentrations. Quantitative PCR and turbidity methods also closely agree thus both methods are likely

accurate in enumerating the total number of MAP. Similarly, Hughes et al. (2001) found a linear relationship between McFarland units and direct MAP counts in a counting chamber, with one McFarland unit being equivalent to 10^8 total cells/mL.

3.7. McFarland units versus plate counts

Using eight MAP isolates including sheep and cattle strains, Elguezabal et al. (2011) found that one McFarland unit was equivalent to 1.3×10^7 CFU/mL by plate counting. In Fernandez et al. (2015), one McFarland unit of a sheep strain was equivalent to 5×10^7 CFU/mL by plate counting. Similar results were obtained for two cattle strains, with estimates by plate count less than half a $\log_{10}$ unit lower than the expected cell counts in McFarland units (Fernandez et al., 2014). For two sheep strains however, this difference was greater, with plate counts 1–2 $\log_{10}$ units lower than expected. The authors assumed that this discrepancy was due to the difficulty of growing sheep strains rather than actual low MAP viability.

3.8. qPCR versus plate counts

For MAP organisms isolated from dairy herds, the Spearman correlation between the Ct value of qPCR and the CFU on Herrold Egg Yolk medium was estimated between -0.66 for faecal pools containing five to several hundred individuals, to -0.76 for fresh environmental samples (Aly et al., 2010). This demonstrates a good correlation between the two methods.

Using three cattle strains of MAP in serial dilutions, Kralik et al. (2012) showed that culture on solid media consistently and significantly underestimated MAP numbers by about two $\log_{10}$ units compared with qPCR. The authors mention that clumping could not be avoided, which is a potential explanation for the difference.

Establishment of the relationship between qPCR and CFU by plate count likely depends on the phase of growth in liquid broth. Elguezabal et al. (2011) showed that qPCR underestimated CFU numbers at low concentrations due to lack of analytical sensitivity. In later stages however, viable counts of CFU underestimated the number of genome equivalents by up to a factor of 1:187 after 142 days of culture. This is probably due to clumping, loss of viability, or dormancy occurring in the late stages of *in vitro* culture. This discrepancy reflects biases in both enumeration methods. It likely results in an underestimate of viable MAP numbers by culture-based methods and an overestimate by qPCR. The exact extent of each bias cannot be quantified precisely. For determining the analytical sensitivity of qPCR, culture-based methods should thus not be used as a reference (Kralik et al., 2012). Instead, a direct count on ZN coloured smears, with oil-immersion microscopy is preferred, since this provides more accurate results for total cell counts in suspensions above the limit of detection for direct microscopy, being thus well suited as a reference for qPCR.

4. Summary

Enumeration of MAP doses is essential for challenge studies, study of the effect of dose on the outcome of infection, and to allow comparison between studies. This review can inform laboratory procedures for MAP enumeration, as well as standard interpretation of inoculum doses from various sources, accounting for biases. A summary of the methods is provided in Table 2.

Some methods can only detect viable (or cultivable) MAP while others can account for both viable and non-viable MAP. Methods enumerating total cells should not be compared with those enumerating viable MAP, and should not be used as a reference for each other. Each enumeration method has inherent bias or shortcomings; the extent of biases varies depending on the isolate or strain (Elguezabal et al., 2011; Shin et al., 2007), while additional biases arise from poor design, such as inappropriate culture medium. Thus, no gold standard exists to

Table 2
Summary of the characteristics of different enumeration methods for MAP.

	Accuracy	Turn-around-time	Cost	Instrumentation required	Affected by clumping	Identify viable* MAP
Solid media culture (CFU)	Poor, highly dependent upon the strain and the use of an appropriate medium	Typically 2-3 months, up to 1 year	Medium	No	yes	Yes
End point titration in liquid culture	Poor especially in clinical samples, but better than plate culture for sheep strains	At least 3 months	High	No	yes	Yes
Time to growth in radiometric Bactec12B (discontinued)	Medium, better when calibrated specifically for the isolate, better in suspensions $> 10^4$/mL	Weeks to months	Medium	Bactec system	Moderately**	Yes
Time to growth in MGIT Paratb	High - Only validated for cattle strains	Within 2 weeks	Medium	Bactec system	To be determined	Yes
Direct microscopic count	Poor, counting chambers for pure culture and stained smears for clinical samples, limit of detection of 10^4/mL	Hours	Low	No	Yes	No
Turbidimetry	Medium to high, suited only for liquid culture*** and high concentration ($> 10^7$/mL)	Hours	Low	Nephelometer or spectrophotometer, except for McFarland units	Yes**	No
Pelleted weight	Medium, better suited for suspensions $> 10^6$ MAP/mL	Hours	Low	No	No	No
qPCR	High, can be used in liquid suspension and clinical specimen, limit of detection 10 MAP/sample (for fecal samples)	Days	high	PCR machine +/- DNA automated extraction system	no	No****

* Viable MAP or most often cultivable MAP. Loss of growing ability or dormancy will lead to underestimates with culture-based methods.
** These methods are also affected by contamination of the culture broth with other bacteria.
*** Turbidimetry is not compatible with the use of a medium containing egg yolk.
**** Although qPCR does not typically distinguish viable and dead cells, recent developments involving the use of propidium monoazide treatment allow the estimation of viable MAP using this technique.

enumerate MAP organisms. Further, enumeration methods are evaluated relative to each other; hence their absolute accuracy remains unknown. Nevertheless, counts variation across enumeration techniques are usually within two log10 units. This is moderate compared with the magnitude of MAP number variation in clinical samples or MAP suspensions in which enumeration is attempted.

To estimate inoculum doses for pathogenesis studies, enumeration of viable MAP using a culture-based method might be more relevant. This would account for any loss of viability occurring during procedures ahead of acquisition of the inoculum suspension. Colony counts on solid media have long been the reference for reporting doses in experimental challenge studies (Elguezabal et al., 2011; Hines et al., 2007; Kralik et al., 2012; Reddacliff, 2002). However, underestimation due to clumping and slow growth makes this method a questionable gold standard for this purpose (Elguezabal et al., 2011). Plate counts are particularly unreliable for sheep strains, for which liquid media are better suited. To enumerate MAP numbers in clinical samples, culture-based methods should be avoided because laboratory processing of the samples involving storage, decontamination, and filtration prior to enumeration causes loss of MAP viability that would affect the result. Methods based on physical properties of MAP, such as turbidimetry or pelleted weights, offer relatively accurate, cheaper and faster alternatives than culture. The highly sensitive direct qPCR method also provides an accurate alternative to culture. In the absence of a reliable reference, several enumeration methods should be attempted in parallel when precise estimates are required, in particular to conduct experimental infection trials.

Conflict of interest statement

The authors declare no conflict of interest.

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