Optimization of a Phage Amplification Assay To Permit Accurate Enumeration of Viable *Mycobacterium avium* subsp. *paratuberculosis* Cells[∇]

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A commercially available phage amplification assay, *FASTPlaqueTB* (Biotec Laboratories, Ipswich, United Kingdom), when used according to the manufacturer's instructions, does not permit accurate enumeration of *Mycobacterium avium* subsp. *paratuberculosis*. The aim of this study was to optimize the phage amplification assay conditions to permit accurate quantification of viable *M. avium* subsp. *paratuberculosis* cells. The burst time for *M. avium* subsp. *paratuberculosis* was initially determined to inform decisions about optimal incubation time before plating, and then other test parameters were altered to evaluate how the correlation between plaque and colony counts was affected. The D29 mycobacteriophage replicates more slowly in *M. avium* subsp. *paratuberculosis* than in *Mycobacterium smegmatis* (used to optimize the commercial test originally), and the mean burst time for four *M. avium* subsp. *paratuberculosis* strains was 210 ± 36.8 min at 37°C compared to 63 ± 17.5 min for *M. smegmatis* mc² 155. To achieve 100% correlation between plaque and colony counts, the optimized phage assay includes the following: (i) resuspension of the samples to be tested in Middlebrook 7H9 broth containing 10% oleic acid-albumin-dextrose-catalase and 2 mM calcium chloride, followed by overnight incubation at 37°C before performance of the phage assay; (ii) a 2-h incubation of the sample with D29 mycobacteriophage before viricide treatment; and (iii) a further 90-min incubation after viricide treatment and neutralization up to the burst time (total incubation time, 210 min) before plating with *M. smegmatis* mc² 155 in 7H9 agar. The optimized phage amplification assay was able to detect 1 to 10 CFU/ml of *M. avium* subsp. *paratuberculosis* in spiked milk or broth within 48 h, as demonstrated by the results of several blind trials.

Mycobacterium avium subsp. *paratuberculosis* is the causative agent of Johne's disease, a chronic enteritis of domestic ruminants, particularly dairy cattle (31). Johne's disease is increasing globally in terms of prevalence in food-producing animals and is a source of significant financial losses annually (13, 20). Due to a possible association with Crohn's disease in humans (4, 22, 27), in the last decade *M. avium* subsp. *paratuberculosis* has become a public health and food safety concern and its possible role as a zoonotic agent has been widely discussed. Milk, dairy products, and water (10, 24) are the most likely vehicles of transmission of *M. avium* subsp. *paratuberculosis* to humans. *M. avium* subsp. *paratuberculosis* has been isolated from retail pasteurized milk (2, 8, 11), and other studies carried out with spiked dairy products showed significant survival rates in cheese up to different stages of ripening (6, 30, 34). A separate study shows that *M. avium* subsp. *paratuberculosis* can survive chlorination of water (36).

Currently the true scale of human exposure to *M. avium* subsp. *paratuberculosis* via food or water is not known, and the main obstacle to providing an accurate assessment of this exposure appears to be the lack of a rapid and specific detection method for viable *M. avium* subsp. *paratuberculosis* cells that

could be used for routine screening purposes. Culture-based detection methods are slow and nonspecific and take weeks to yield colonies. Moreover, the isolation of *M. avium* subsp. *paratuberculosis* from food or other matrices requires lengthy chemical decontamination procedures to suppress the growth of competitive microorganisms. However, these treatments also have some adverse effect on the viability of *M. avium* subsp. *paratuberculosis* cells present, which reduces the sensitivity of the method (7). A quicker detection approach is provided by molecular methods such as PCR. PCR assays, consisting of the amplification of sequences such as the IS900 (12), F57 (23), and ISMap02 sequences (29), allow for rapid and specific detection of *M. avium* subsp. *paratuberculosis* but cannot provide differentiation between the presence of viable and nonviable organisms. Due to difficulties in extracting cells from the matrix and the presence of contaminants that inhibit DNA amplification (14), even reverse transcription-PCR assays showing amplified IS900 mRNA are difficult to apply as routine tests. A promising alternative to conventional culture seems to come from phage amplification technology, as applied in the commercially available *FASTPlaqueTB* (FPTB) assay (Biotec Laboratories Ltd., Ipswich, United Kingdom). This assay has been used as a low-cost, rapid approach for detection of viable *M. tuberculosis* cells in sputum (25) as well as for detection of *M. avium* subsp. *paratuberculosis* in milk (1, 32). The method exploits the ability of D29 mycobacteriophage to replicate within and subsequently lyse only viable cells of several *Mycobacterium* spp. (26). Products of amplification following infection can be viewed as lysed areas (plaques), which

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TABLE 1. Bacteria and mycobacteriophage included in the study

Strain or mycobacteriophage	Origin
<i>M. avium</i> subsp. <i>paratuberculosis</i>	
ATCC 19698.....	American Type Culture Collection
NCTC 8578.....	National Collection of Type Cultures
B2.....	Bovine isolate; Veterinary Service for Northern Ireland
796PSS.....	Pasteurized milk isolate ^a
806R.....	Raw milk isolate ^a
<i>M. smegmatis</i> mc ² 155.....	Gift from Ruth McNerney ^b
D29.....	Gift from Ruth McNerney ^b

^a Originally isolated by Irene Grant, Queen's University, Belfast, Northern Ireland (11).

^b London School of Hygiene and Tropical Medicine, London, United Kingdom.

can be recorded after 24 h on indicator plates prepared with fast-growing *Mycobacterium smegmatis*.

Stanley et al. (32) were the first to report that the D29 mycobacteriophage could infect *M. avium* subsp. *paratuberculosis* in studies involving the commercial FPTB assay to enumerate *M. avium* subsp. *paratuberculosis* in milk. Altic et al. (1) used the FPTB assay in UV inactivation studies but observed poor correlation between plaque counts (PFU/ml) and colony counts (CFU/ml). However, UV inactivation trends indicated by PFU/ml and CFU/ml counts were similar, suggesting that test parameters (e.g., rate of infection of *M. avium* subsp. *paratuberculosis* by D29, rate of D29 amplification within *M. avium* subsp. *paratuberculosis*, incubation time before burst, etc.) were far from optimal for *M. avium* subsp. *paratuberculosis* enumeration. The commercial FPTB test was developed with *M. smegmatis* (A. Trollip, Biotec Laboratories, personal communication), which is a much faster growing *Mycobacterium* sp. than *M. avium* subsp. *paratuberculosis*.

The aim of the present study was to optimize the phage amplification assay to achieve accurate enumeration of viable *M. avium* subsp. *paratuberculosis* cells. The conventional method theoretically requires leaving the infection as long as the latent period and plating the sample before phage release. As there is no available information about the replication rates of D29 phage within *M. avium* subsp. *paratuberculosis*, this study was carried out to (i) investigate the optimal conditions for D29 phage infection of *M. avium* subsp. *paratuberculosis* cells, (ii) determine the minimal time for D29 incubation with *M. avium* subsp. *paratuberculosis* to produce extracellular phage (burst time), (iii) evaluate whether the incubation time before viricide treatment needs to be extended over 1 h when testing *M. avium* subsp. *paratuberculosis*, and (iv) assess the impact of changing these parameters on the correlation between PFU and corresponding CFU counts per ml.

MATERIALS AND METHODS

Culture of bacterial strains. Five strains of *M. avium* subsp. *paratuberculosis* were included in this study as detailed in Table 1. *M. avium* subsp. *paratuberculosis* strains were grown in a shaker incubator for 4 to 6 weeks at 37°C to stationary phase in Middlebrook 7H9 broth containing 10% oleic acid-albumin-dextrose-catalase (OADC) supplement (both from Difco) and 2 µg/ml mycobactin J (Synbiotics Europe SAS, Lyon, France). *Mycobacterium smegmatis* mc² 155 was cultured to stationary phase (2 to 3 days at 37°C) in the same medium without the addition of mycobactin J. Tween 80 was not added to 7H9 broth during these studies, as it is known to interfere with phage adsorption.

Propagation of D29 mycobacteriophage. D29 mycobacteriophage was propagated in agar plates containing *M. smegmatis* mc² 155 as described by McNerney et al. (18). Briefly, two or three Middlebrook 7H9 agar plates containing around 100 to 200 plaques were flooded with 10 ml 7H9 broth and incubated overnight at 4°C. The broth containing phage particles was recovered and centrifuged (2,500 × g for 10 min), and supernatant was filtered through 0.22-µm Millex GP Millipore Express PES membrane filter units (Millipore Corporation, MA) and stored at 4°C. The number of phage present in this stock culture was determined by titration, which involved dilution in 7H9 broth and plating in petri dishes along with 1 ml *M. smegmatis* mc² 155 culture and molten 7H9 agar. The D29 stock solution for use in further experiments was standardized to a concentration of 10⁹ PFU/ml and stored at 4°C until use.

Conventional phage assay. Detection of viable cells of *M. avium* subsp. *paratuberculosis* was first evaluated by the FPTB assay performed according to the manufacturer's instructions (Biotec Laboratories Ltd., Ipswich, United Kingdom). Bacterial samples containing around 10⁵ CFU *M. avium* subsp. *paratuberculosis*/ml were resuspended in 1 ml FPTB Media Plus. After addition of Ac-tiphage (100 µl, containing 10⁸ PFU mycobacteriophage) samples were incubated for 60 min at 37°C and then treated with 100 µl of Virusol (virucidal solution), which was left to act for 5 min at room temperature. Virusol activity was then neutralized by adding 5 ml of FPTB Media Plus, and samples were mixed with 1 ml sensor cells (10⁸ CFU *M. smegmatis*), poured into petri dishes containing 5 ml molten FPTB agar (55°C), and allowed to set. Plates were incubated overnight (18 h) at 37°C, and plaques produced confirmed the presence of mycobacteria in the test sample.

Colony count determination. Throughout this study colony counts (CFU/ml) for comparison with plaque counts were obtained by plating dilutions of *M. avium* subsp. *paratuberculosis* cultures on Herrold's egg yolk medium containing 2 µg/ml mycobactin J (HEYM) and incubating plates wrapped in Duraseal laboratory sealing film (Diversified Biotech, MA) at 37°C for 4 to 6 weeks before counting colonies.

Determination of burst time for *M. avium* subsp. *paratuberculosis* and *M. smegmatis* mc² 155. Aliquots (3 ml) of broth cultures of four strains of *M. avium* subsp. *paratuberculosis* and *M. smegmatis* mc² 155, grown to stationary phase in Middlebrook 7H9 broth supplemented with 10% OADC and 1 mM CaCl₂ (and mycobactin J in the case of *M. avium* subsp. *paratuberculosis*), were decuppled by vortexing with five 3-mm sterile glass beads (VWR International Ltd., England) for 2 min before addition of D29 phage (10⁸ PFU/ml). Broth cultures were incubated without shaking at 37°C, and lysis of cells, as a result of phage replication, was monitored by the measurement of optical density at 600 nm (OD₆₀₀) every 15 min (21) with a WPA CO8000 cell density meter (SISLAB, Italy). The experiment was repeated in triplicate, and the mean burst times for *M. avium* subsp. *paratuberculosis* and *M. smegmatis* mc² 155 were calculated from the point of inflection of the graphs obtained by plotting the change in OD against incubation time.

Effect of extended incubation time before viricide treatment on correlation between PFU and CFU. As the previous experiment (see Fig. 1) showed that the burst time for *M. avium* subsp. *paratuberculosis* occurred later than for fast-growing *M. smegmatis*, we first evaluated if the correlation between PFU/ml and CFU/ml could be improved by extending the incubation time with D29 phage until the burst time. One-milliliter samples of Middlebrook 7H9 broth containing 10⁷ CFU/ml *M. avium* subsp. *paratuberculosis* were incubated with D29 phage (10⁸ PFU/ml) at 37°C for 60, 90, 120, 150, 180, 210, and 240 min before treatment with 100 µl of freshly prepared 100 mM ferrous ammonium sulfate (FAS; Sigma) at room temperature for 5 min, neutralization with 5 ml 7H9 broth, and plating with *M. smegmatis* mc² 155 in 7H9 agar. Plaque counts (PFU/ml) were monitored, and a reduction in PFU/ml after the longest incubation times was taken as indicative of inactivation of phage released from *M. avium* subsp. *paratuberculosis* cells. PFU counts were compared with colony counts of the spiked broth culture on Herrold's egg yolk medium, and the correlation between PFU/ml and CFU/ml was calculated. This experiment was repeated three times for both ATCC 19698 and NCTC 8578 and once for B2, 796PSS, and 806R.

Optimization of other phage assay conditions. Further aspects of the commercial test protocol were modified as described below. Samples containing 10⁵ CFU *M. avium* subsp. *paratuberculosis*/ml were tested in all experiments, and colony counts were carried out for comparison with plaque counts by plating appropriate dilutions on HEYM.

(i) Optimization of CaCl₂ concentration for phage infection/production. *M. avium* subsp. *paratuberculosis* cells were resuspended in Middlebrook 7H9 broth containing 10% OADC supplemented with 1 mM, 2 mM, 4 mM, 5 mM, 10 mM, or 20 mM CaCl₂ and then inoculated with D29 mycobacteriophage (10⁸ PFU/ml) and incubated for 15 min at 37°C before treatment with 100 µl of 100 mM FAS for 5 min at room temperature. After neutralization of FAS by the addition of 5

FIG. 1. Mean burst times (indicated by arrows) obtained for *M. avium* subsp. *paratuberculosis* (four strains; diamonds) and *M. smegmatis* mc² 155 (squares) determined by monitoring OD₆₀₀ of broth cultures after addition of D29 mycobacteriophage and incubation at 37°C.

ml of the suspension broth, samples were incubated for up to 60 min before plating with *M. smegmatis* mc² 155 in 7H9 agar. This experiment was replicated three times. Once the optimal CaCl₂ concentration had been determined, the effect of extended incubation times (1 h, 2 h, 3 h, or overnight at 37°C) in the presence of this CaCl₂ concentration before performing the phage assay was evaluated. This experiment was replicated twice. Calcium chloride concentration and incubation time yielding maximum PFU/ml were taken as indicative of the most appropriate test conditions.

(ii) **Optimization of total incubation time before plating.** To determine the length of the latent period for D29 phage infection of *M. avium* subsp. *paratuberculosis* cells and the burst time, the phage assay was carried out by performing FAS treatment after 15 min, neutralizing FAS by addition of 7H9-OADC-2 mM CaCl₂, and then incubating for a further 20, 60, 100, 140, 180, 220, 260, and 300 min at 37°C. This meant total incubation times after D29 infection of 40, 80, 120, 160, 200, 240, 280, and 320 min before plating with *M. smegmatis* mc² 155 in molten 7H9 agar. Dilutions were prepared, if appropriate, in 7H9-OADC-2 mM CaCl₂ before plating. The incubation time at which PFU counts plateaued was considered the burst time for *M. avium* subsp. *paratuberculosis*.

(iii) **Optimization of infection time before viricide treatment.** Seven 1-ml aliquots of the same 4-week-old culture of *M. avium* subsp. *paratuberculosis* suspended in 7H9-OADC-2 mM CaCl₂ were inoculated with D29 phage (10⁸ PFU/ml) and incubated at 37°C to allow infection. After different incubation times (60, 90, 120, 150, 180, 210, and 240 min) viricide treatment was performed with 100 µl freshly prepared 100 mM FAS for 5 min at room temperature. FAS was neutralized by addition of 5 ml 7H9-OADC-2 mM CaCl₂ before dilutions were prepared, as appropriate, in 7H9-OADC-2 mM CaCl₂. Samples were then poured into petri dishes containing 1 ml *M. smegmatis* mc² 155 culture and 5 ml molten 7H9 agar. Plates were swirled to mix sample with agar before being allowed to set and then incubated overnight at 37°C. Plaques were counted, and the incubation time resulting in maximum PFU/ml was determined. This experiment was repeated twice for *M. avium* subsp. *paratuberculosis* strain ATCC 19698.

Blind trials. To determine the lowest number of *M. avium* subsp. *paratuberculosis* cells detectable by the optimized phage assay, several blind trials were performed. Each trial consisted of 12 7H9-OADC-2 mM CaCl₂ broth or ultra-heat-treated whole-milk suspensions (1 ml) containing different numbers of *M. avium* subsp. *paratuberculosis* CFU/ml (three with 10² to 10³ CFU/ml, three with 10 to 10² CFU/ml, three with 1 to 10 CFU/ml, and three unspiked) set up by a colleague. The operator was blind to the quantity of cells inoculated into each sample until the results were obtained. Broth samples were incubated at 37°C overnight before the optimized phage assay. Milk samples were centrifuged (16,800 × g for 15 min) and resuspended in 1 ml 7H9-OADC-2 mM CaCl₂ before being incubated overnight at 37°C and processed through the optimized phage assay.

Statistical analysis. The statistical significance of differences between colony and plaque counts was assessed by a paired *t* test (Instat 3; GraphPad, La Jolla, CA); differences for which *P* was <0.05 were considered significant. Correlation

between plaque and colony counts obtained from the blind trials was assessed using linear regression analysis (Microsoft Excel).

RESULTS

Detection of the mean burst time of *M. avium* subsp. *paratuberculosis*. *M. avium* subsp. *paratuberculosis* broth cultures were infected with D29 phage and incubated at 37°C, and the cell density at 600 nm was measured every 15 min. As expected, cell lysis, resulting in the release of new phage particles, produced a progressive decline of the OD₆₀₀ value (Fig. 1). The length of the latent period of D29 phage in *M. avium* subsp. *paratuberculosis* cells was longer than that for *M. smegmatis*. As shown in Fig. 1, the mean burst times determined for *M. smegmatis* mc² 155 and four strains of *M. avium* subsp. *paratuberculosis* were 63 ± 17.5 min and 221.6 ± 36.8 min, respectively. There was some variation in burst time between *M. avium* subsp. *paratuberculosis* strains: ATCC 19698, 215.0 ± 7.1 min; NCTC 8578, 172.5 ± 22.5 min; 806R, 260.0 ± 10.0 min; and 796PSS, 242.5 ± 32.5 min. Differences in the age of culture tested (which ranged from 4 to 10 weeks) probably contributed to the variability in burst times observed for different strains, as 4-week-old *M. avium* subsp. *paratuberculosis* broth cultures showed a mean burst time shorter than that shown by cultures cultivated for longer periods (data not shown). The change in OD over time of D29 phage-infected *M. avium* subsp. *paratuberculosis* cultures was slower than that of D29 phage-infected *M. smegmatis* mc² 155 cultures, with the total decrease in OD consistently <30% (Fig. 1). Similar trends were observed with another slow-growing *Mycobacterium* sp. (*Mycobacterium bovis* BCG NCTC 5692), probably due to the high number of clumps present, which were more difficult to disperse than those produced by fast-growing *M. smegmatis*. This may have affected the OD reading. The non-optimal conditions for D29 infection of *M. avium* subsp. *paratuberculosis* may have led to incomplete burst and contributed to the limited reduction in OD observed.

Conventional phage assay. To confirm the length of latent period obtained from burst time experiments, 4-week-old *M.*

FIG. 2. Effect of (A) different calcium chloride concentrations (for 30 min) and (B) preincubation times with optimal calcium chloride concentration (2 mM) on plaque production after D29 infection of *M. avium* subsp. *paratuberculosis* ATCC 19698. Dashed lines indicate corresponding mean colony counts (log₁₀ CFU/ml) for samples tested.

avium subsp. *paratuberculosis* cultures were processed through the conventional phage assay except that viricide treatment was performed after different incubation times after D29 infection up to 240 min. It was expected that PFU/ml would increase progressively during the latent period until a reduction after the burst time, due to FAS inactivation of phage released from lysed mycobacterial cells. PFU/ml did not increase significantly with increased incubation time ($P > 0.05$). The release of new-generation phage was earlier than expected (after 1 h of incubation), and only a slight increase in phage number was observed for samples incubated for longer periods. The comparison between plaque and colony counts indicated that PFU/ml was consistently 1.5 log₁₀ lower than CFU/ml, signifying that only a small percentage of viable cells (around 1%) had been successfully infected. In contrast to what was observed with *M. smegmatis*, *M. avium* subsp. *paratuberculosis* produced plaques which appeared smaller and extremely dissimilar. These were interpreted as indicative of failed or unproductive infections.

Optimization of phage assay conditions. (i) Optimal CaCl₂ concentration for plaque production. The ability of CaCl₂ to improve the rates of D29 mycobacteriophage adsorption onto

FIG. 3. Lysis kinetics of D29 phage-infected *M. avium* subsp. *paratuberculosis*. Samples were incubated overnight in 2 mM CaCl₂ at 37°C before the phage assay. Infection was allowed to proceed for 15 min before viricide treatment. The time of incubation after D29 infection was then extended up to 320 min. The corresponding mean colony count (log₁₀ CFU/ml) for samples tested is indicated by the dashed line.

M. avium subsp. *paratuberculosis* cells was investigated, and results are presented in Fig. 2A and B. The efficiency of infection was found to be slightly improved when CaCl₂ concentration was increased from 1 mM to 2 mM, as samples resuspended in 2 mM CaCl₂ showed the highest plaque count (Fig. 2A). An improvement in the appearance of lysis areas (plaques), which were more uniform and bigger than those recorded from samples processed with lower concentrations of CaCl₂, was also noted. No additional benefit was observed by using CaCl₂ concentrations higher than 2 mM. Once 2 mM CaCl₂ had been identified as the optimal concentration for phage adsorption, we evaluated whether or not a longer incubation time before phage addition was needed to allow the CaCl₂ to act on the *M. avium* subsp. *paratuberculosis* cell wall. Figure 2B demonstrates that plaque production improved significantly ($P < 0.001$) if *M. avium* subsp. *paratuberculosis* cells were incubated overnight with 2 mM CaCl₂. In fact the plaque count recorded was around 1.5 log₁₀ higher than that recorded from samples incubated for shorter time periods or at 4°C overnight.

(ii) Optimal total incubation time before plating. Results obtained from experiments to determine the optimal incubation time with D29 phage before plating are shown in Fig. 3. The difference between plaque and colony counts was found to decrease as incubation time increased from 1.33 log₁₀ at 40 min to 0.33 log₁₀ at 240 min ($P < 0.001$); however, 100% correlation between PFU/ml and CFU/ml counts was not achieved by changing just this test parameter, suggesting that other aspects of the test besides the incubation time still needed to be optimized.

(iii) Optimal infection time before viricide (FAS) treatment. Incubation time with D29 before viricide treatment was extended up to 240 min. Figure 4 shows that the highest number of plaques was found in samples infected with D29 phage for 2 h ($P < 0.001$) and incubated for a further 90 min before plating (i.e., total incubation time of 210 min). A progressive decline in plaque production was observed in samples which

FIG. 4. Impact of infection time before virucide treatment on correlation between $\log_{10}$ PFU/ml and CFU/ml counts. *M. avium* subsp. *paratuberculosis* cells were incubated overnight in 2 mM CaCl_2 at 37°C before a phage assay with a total incubation time of 210 min (i.e., 120 min before virucide treatment plus extended incubation of 90 min) was performed. The corresponding mean colony count ($\log_{10}$ CFU/ml) for samples tested is indicated by the dashed line.

the virucide treatment was performed after an incubation time longer than 2 h.

Detection sensitivity of the optimized phage assay. Results of the blind trial with spiked broth and milk showed that the optimized phage assay permits detection of low numbers of *M. avium* subsp. *paratuberculosis* cells (Fig. 5A and B, respectively). Plaques were recorded from spiked broth and milk samples containing less than 10 CFU *M. avium* subsp. *paratuberculosis*/ml. Mean differences between PFU and CFU counts were $0.45 \log_{10}$ and $0.23 \log_{10}$ for spiked broth and milk, respectively, with PFU/ml counts being consistently slightly higher than CFU/ml counts.

DISCUSSION

This study was undertaken to evaluate whether the poor correlation between PFU/ml and CFU/ml observed by Altic et al. (1) when the commercially available FPTB phage amplification assay was applied to enumerate *M. avium* subsp. *paratuberculosis* cells could be improved and the detection sensitivity of the assay enhanced. As the time required for D29 phage to replicate in bacterial cells (length of the latent period) and the time taken to complete release of new phage particles (burst time) are related to host generation times, the replication rate of D29 phage in *M. avium* subsp. *paratuberculosis* was initially studied to enable the adaptation of the test conditions to biological features of this *Mycobacterium* sp. rather than *M. smegmatis*. Since no information was available in the literature about the replication cycle of D29 in *M. avium* subsp. *paratuberculosis*, the burst time was first determined by measurement of OD over time after D29 infection. The mean burst time obtained for four different *M. avium* subsp. *paratuberculosis* strains was 210 ± 36.8 min, which is considerably longer than that for fast-growing *M. smegmatis* mc² 155 (63 ± 17.5 min). A smaller reduction in OD over time for *M. avium* subsp. *paratuberculosis* than for *M. smegmatis* was observed (15% compared to 55%), suggesting that a lower proportion of cells had been infected. This finding was confirmed by the results ob-

FIG. 5. Relationship between $\log_{10}$ colony and plaque counts of *M. avium* subsp. *paratuberculosis* obtained from blind trials carried out with (A) spiked 7H9 Middlebrook broth and (B) spiked whole milk.

tained from the FPTB assay. Even if infection was allowed to proceed for longer than the 60 min recommended in the commercial assay, only a slight increase of PFU/ml ($0.5 \log_{10}$) was observed over time; however, this value was still substantially lower ($1.5 \log_{10}$) than the corresponding CFU/ml. This result suggested that a high percentage of bacteria (perhaps due to clumping) were not involved in phage infection and that only a small percentage of cells was detectable by the nonoptimized phage assay. Cultures of *M. avium* subsp. *paratuberculosis* are likely to contain cells in different metabolic states, and it is possible that cells aggregated in clumps had lower sensitivity to phage infection, leading to abortive infection. Since it is not possible to prevent clump formation by using polyoxyethylene-sorbitan (Tween 80) in the 7H9 medium, as it affects phage adsorption (35), the subsequent investigation sought to determine how to increase the sensitivity of *M. avium* subsp. *paratuberculosis* cells to phage infection.

A number of published phage papers (3, 5, 16, 28, 35) report that the efficiency of phage infection in *Mycobacterium* spp. is improved by the incorporation of a low level of available calcium supplied as a part of a new liquid medium prior to infection, although the exact action of calcium chloride has not yet been clarified. *Mycobacterium* spp. produce lipid substances which accumulate progressively onto the surface of the cell

wall (15), potentially masking the receptors which phage adsorb onto. It is possible that calcium chloride contained in the buffer may enhance the removal of the masking substances from the surface of the cell wall. Moreover, it is reported that Ca^{2+} ions are required during the injection of phage DNA in host cells, probably stimulating the ATP hydrolyzing activity associated with the contraction of the tail structures (16). We observed at least a twofold increase in plaque production when 2 mM calcium chloride was present in the suspending broth compared to plaque production when lower (1 mM) and higher (4, 5, 10, and 20 mM) calcium chloride concentrations were used (Fig. 2A). The optimal calcium chloride concentration for *M. avium* subsp. *paratuberculosis* is higher than that supplied in the FPTB kit (A. Trollip, Biotec Laboratories, personal communication). In addition, phage infection (evidenced by increased plaque count) was found to increase significantly when the calcium chloride was allowed to act overnight before phage was added (Fig. 2B). This finding suggests that calcium chloride activity is possibly related to replication cycles of the host bacterium and confirms the observation of David et al. (5) that better adsorption rates are achieved if *Mycobacterium* spp. are washed and resuspended in new calcium chloride-containing broth for one or two division times before phage assay. Overnight incubation (18 to 20 h) would equate to approximately one generation time for *M. avium* subsp. *paratuberculosis* in Middlebrook 7H9 broth at 37°C (I. Grant, unpublished data; 17).

Having established the optimal CaCl_2 conditions for phage infection of *M. avium* subsp. *paratuberculosis*, phage release over time was monitored to determine the optimum incubation time after D29 infection before plating. Viricide treatment was performed after 15 min (which assumed rapid phage adsorption and penetration), and the time after D29 infection was extended up to 320 min. A significant increase in PFU/ml count ($1.5 \log_{10}$) was observed as incubation time increased up to 240 min (Fig. 3), when PFU/ml counts plateaued. This result confirmed what was previously observed in burst time experiments. Lysis of the majority of *M. avium* subsp. *paratuberculosis* cells occurred between 200 and 240 min. The time of phage release depends of the metabolic state of host cells, and a high percentage of *M. avium* subsp. *paratuberculosis* cells may require a longer time to replicate and release the new phage progeny if, for example, they are in clumps or are less sensitive to phage infection. The correlation between $\log_{10}$ PFU/ml and $\log_{10}$ CFU/ml counts at the burst time (220 min) was 95% compared to 80% when the standard phage assay conditions (total incubation time, 60 min) were applied.

In order to further improve the correlation between PFU and CFU counts, subsequent experiments focused on optimizing the incubation time with D29 before viricide treatment. In the commercial phage assay, *M. smegmatis* needs to be incubated with phage for 60 min before viricide treatment to produce 100% correlation between PFU/ml and CFU/ml counts, so times longer than 60 min for *M. avium* subsp. *paratuberculosis* were studied. To enter into host cells, bacteriophage particles need to attach to the cellular receptor on the surface of bacteria and inject their genetic material into the cell to be replicated. It is likely that the time necessary for attachment and penetration is host related. The maximum number of plaques was obtained from cell suspensions which had been

infected for 2 h before viricide treatment (Fig. 4). It is possible that decreased efficiency of the cell wall mechanism allowing DNA injection (due to clumps or less-sensitive cells) or the morphology of clumps (i.e., less surface area of the cell visible, resulting in decreased availability of receptor sites for infection) could extend the time required for optimal phage absorption and explain this finding.

On the basis of the results presented it is concluded that to accurately enumerate small numbers of *M. avium* subsp. *paratuberculosis* cells, some aspects of the commercial FPTB assay (Biotec Laboratories, Ipswich, United Kingdom) need to be changed. In order to achieve close to 100% correlation between PFU/ml and CFU/ml counts when *M. avium* subsp. *paratuberculosis* is tested, the sample should be resuspended in 7H9-OADC broth containing 2 mM calcium chloride and incubated overnight at 37°C before D29 phage particles are added. Infection with D29 mycobacteriophage should proceed for 2 h before FAS (viricide) treatment; then, because release of new phage particles in *M. avium* subsp. *paratuberculosis* is not synchronous, as in fast-growing mycobacteria (*M. smegmatis*), further incubation up to a total time after D29 infection of 210 min is required to achieve phage release from a higher percentage of cells. This optimized phage assay is a promising tool providing rapid and sensitive enumeration of viable *M. avium* subsp. *paratuberculosis* cells. Blind trials confirmed that the optimized phage assay can detect fewer than 10 CFU/ml in spiked broth or milk within 48 h and would have application for inactivation studies where pure *M. avium* subsp. *paratuberculosis* cultures were being tested. However, it could not be applied on its own for testing naturally infected samples as, due to the wide host range of the D29 mycobacteriophage, other environmental mycobacteria could be infected and form plaques. In order to improve the specificity of the phage assay for *M. avium* subsp. *paratuberculosis*, it would need to be combined with plaque PCR as described by Stanley et al. (32) or potentially coupled with specific immunomagnetic separation techniques already developed for this pathogen (9, 19, 33).

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