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Flow cytometric assessment of the survival ratio of *Francisella tularensis* in aerobiological samples

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Abstract

Survival of microorganisms in aerobiological samples is often assessed by a survival ratio (SR), which is the ratio between the viable or metabolic active number (MA) of microorganisms to the total number (TOT) of microorganisms. A method to determine survival ratios with flow cytometry was developed for *Francisella tularensis*, the causative agent of tularemia. *F. tularensis* is a fastidious bacteria that can be transmitted by aerosol and constitute as such a valid model organism for aerobiological studies. The total number of *F. tularensis* cells was determined by specific targeting with monoclonal antibodies and detected by phycoerytrine (PE) conjugated secondary antibodies. The metabolic active part of the targeted *F. tularensis* cells was quantified by staining with rhodamine 123 (Rh123). Application of the presented method showed higher precision compared to an earlier developed method for survival ratios, achieved with plate count (VC) and Coulter Counter (CC) measurements. The coefficient of variation between samples for the new method was below 5% for the survival ratio. Comparison of VC yield with MA yield showed consistently higher values for MA. The survival ratios of *F. tularensis* in samples taken before and after aerosolisation were analysed. SR for *F. tularensis* determined with the new method decreased approximately 19% whereas SR determined with VC and CC decreased 62% after passage through aerosol state.

Keywords: *Francisella tularensis*; Flow cytometry; Survival ratio; Aerosol; Rhodamine 123; Monoclonal antibody

1. Introduction

To assess the biological hazard posed from airborne microorganisms to human health, it is important to use methods that reflect the metabolic activity of the microorganisms in the air. The capability of causing an infection or producing toxins is dependent on the metabolic activity. It is therefore also crucial to maintain the integrity of an air sample all the way from collection, through handling to final analysis. This is described as the sampler's ability to keep the microorganism intact, i.e. preservation efficiency, and is best defined by the relative survival efficiency compared with a reference sampler [1]. In laboratory studies of aerosols, survival is most com-

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monly defined as a survival ratio, which is the ratio between the viable or the metabolic active number to the total number of microorganisms. Traditionally, viable count (VC) relies on techniques developed decades ago, such as plate count and the most-probably number technique [2,3]. However, it is a well-known fact that many microorganisms do not grow under standard conditions used in the laboratory [4]. Furthermore, in determining survival ratios [5], plate count was the method with the lowest precision. Consequently, there is a need to replace the traditional plate count or culture techniques with more precise and non-biased methods. There exist several methods for estimation of total number of microorganisms. However, in a comparative study of different methods to determine the total number of bacteria in aerobiological samples it was found that Coulter Counter (CC) analysis was preferred because of its rapidity, high precision and yield [5]. A chief limitation with CC is, however, that it cannot be used for detection of microorganisms in environmental samples, because microorganisms cannot be distinguished from inert particles. However, the use of flow cytometry for counting particles in a liquid with analysis based on light beam analysis, instead of voltage changes as in CC, is a technique with great promises for identification of microbial populations in environmental samples [6].

With the aim to study the survival of the small Gram-negative bacterium *F. tularensis*, the causative agent of tularemia, in aerosol phase we have in this paper applied and evaluated a flow cytometric method for measurement of survival ratio. The bacterium *F. tularensis* is highly infectious and as few as ten bacteria are needed to cause infection [7]. Respiratory tularemia is transmitted through inhalation of the causative agent and the largest outbreak reported in Sweden of the disease was due to inhalation of contaminated airborne dust [8].

The flow cytometric method of analysis (FC) was based on staining and use of monoclonal antibodies. The total number of *F. tularensis* cells was determined by specific targeting with monoclonal antibodies which was detected by PE-conjugated secondary antibodies. The metabolic active part of the targeted *F. tularensis* cells was quantified by staining with Rh123. This method was compared to a previously studied method for determining survival ratios, based on plate count and CC measurements [5], and applied on samples taken from aerosolised *F. tularensis*.

2. Materials and methods

2.1. Bacteria and reagents

Francisella tularensis LVS, live vaccine strain, was obtained from US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA. The source for the *Escherichia coli* strain used was ATCC 25955. Monoclonal antibodies specific for *F. tularensis*, designated FT14, were kindly provided by CBDE, Porton Down, Salisbury, UK.

2.2. Growth of F. tularensis and E. coli

Modified Thayer-Martin agar plates containing Gc medium base (36 g/l; Difco Laboratories, Detroit, MI), haemoglobin (10 g/l; Difco), and IsoVitaleX (10 mg/l; BBL Microbiology Systems, Cockeysville, MD), was used for viable counts. After spreading of the bacteria, the agar plates were incubated for two days at 37°C in 5% CO_2. For growth of *F. tularensis* LVS in liquid media Scharer medium [9] was used. After inoculation of approximately 10^6 cells ml^{-1} the cultures were agitated (150 rpm) at 37°C for 18 h. *E. coli* was grown in LB media or agar plates thereof [10] at 37°C overnight.

2.3. Staining and labelling procedure

The bacterial samples were stained with Rh123 according to the method of Kaprelyant and Kell [11] with some minor modifications and analysed by flow cytometry. The bacteria were harvested and resuspended in phosphate buffer saline (PBS) at a density of approximately 10^8 cells ml^{-1}. One ml of this suspension was centrifuged at $10\,000 \times g$ for 7 min and washed three times in TE-buffer [12]. After the last centrifugation, the pellet was resuspended in 900 μl of M9-medium [12]. Next, Rh123 (Sigma Chemical Co., St. Louis, MO, USA) was added to a final concentration of 10.4 mM and the bacterial cell suspension was agitated for 15 min at room temperature in the dark. The sample was then

washed twice in PBS by consecutive centrifugations, as described above, and finally resuspended in 900 μl of PBS. In some experiments, the Rh123 fluorescence was analysed in the flow cytometer at this stage. Following staining with Rh123, 100 μl of the cell suspension was withdrawn and 10 μl of monoclonal antibodies (FT14), diluted according to Fulop et al. [13], was added. The antibodies were allowed to react with the Rh123 stained *F. tularensis* (or *E. coli*) for 20 min at room temperature in the dark. Next, the sample was washed twice in PBS by consecutive centrifugations, as described above, and resuspended in 30 μl of PE-conjugated, affinity pure $F(ab)_2$ fragment goat anti-mouse IgM antibody (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA) solution, diluted 1:50 and prepared according to the manufacturer's instructions. The PE-conjugated anti-antibodies were allowed to react with FT14 coated *F. tularensis* for 15 min at room temperature in the dark. After another washing, the pellet was resuspended in 450 μl of PBS prior to analysis with flow-cytometry. The losses of *F. tularensis* cells after each centrifugation step during the staining and labelling were determined by plate count ($n = 17$). The cumulative loss factor, taking into account all centrifugation steps, amounted to 13.2 ± 1.7 (mean and standard error of mean).

Negative control samples were prepared and used as reference samples for the Rh123 staining procedure of *F. tularensis*. These reference samples included unstained viable *F. tularensis* and dead cells of *F. tularensis*. To obtain dead cells of *F. tularensis*, the bacteria were grown on modified Thayer-Martin agar plates for two days, whereupon drops of 37% formaldehyde were placed on the lids and the plates were stored in an inverted position at room temperature for at least 70 h. These cells were then stored at 4°C. The inability to form colonies on plates and absence of metabolic activity were confirmed in each experiment.

2.4. Flow cytometer analyses

Measurements of Rh123 fluorescence and antibody binding to the bacteria was done using a flow cytometer (FACSort, Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with an argon laser with a 488-nm primary emission line. Rh123 fluorescence was detected using the primary photomultiplier tube with a band pass width of 530 ± 30 nm. For the PE-labelling the secondary photomultiplier tube (585 ± 42 nm) was used. The instrument settings for Rh123+PE analysis were for FL1 and FL2 725 V and 412 V with a compensation of 8% FL2 and 24% FL1, respectively. The threshold trigger was adjusted to 25 and the measuring time was 30 s with a medium flow of 30 μl. Statistics of the results was compared with CC measurement. Settings for the CC has been described previously [5].

2.5. Aerosol system

The aerosol system used for aerosol samples consisted of a constant output atomiser, a conditioning system (drying and neutralising) and a dynamic aerosol chamber [14]. The samples were collected in PBS by an All Glass Impinger-30 (AGI-30). *F. tularensis* grown in Scharer medium was used for generation. Two independent aerosolisation experiments were conducted and analysed.

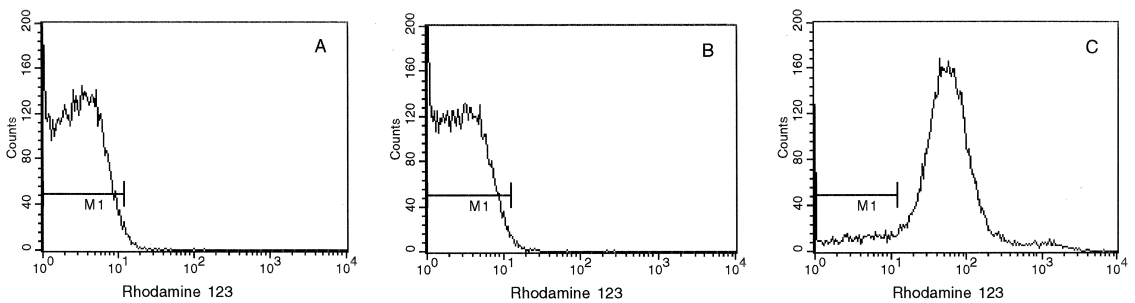

Fig. 1. Rh123 fluorescence vs. counts for: A: stained metabolic inactive/dead cells; B: unstained viable cells; and C: stained viable cells.

Table 1
Metabolic activity of *F. tularensis* cells measured by viable count and by flow cytometry

	VC[a] (CFU[b]/ml)	MA (no./ml)	FC_{TOT}[c] (no./ml)	SR MA/FC_{TOT} (%)
Viable stained cells	3.08×10^5	3.05×10^6	3.30×10^6	92
Dead stained cells	0	4.95×10^3	2.28×10^6	0.2

As viable cells, diluted samples from a culture of *F. tularensis* were used. Dead *F. tularensis* cells were obtained by treatment with formaldehyde. Values are given as means ($n=2$).
[a]Viable count performed as plate count.
[b]Colony forming units.
[c]Total number by flow cytometry.

2.6. Statistical analysis of data

Statistical analysis of the results for determination of the total number was performed with T-test for equality of means. The software used for analysis of the results was SPSS (SPSS Inc., IL, USA).

3. Results

3.1. Analyses of Rh123 stained F. tularensis cells

The fluorescent dye Rh123 is concentrated by microbial cells in an energy-dependent fashion and the accumulation of Rh123 can rapidly be assessed by flow cytometry [11,15,16]. Hence, staining with Rh123 and subsequent flow cytometric analysis is one approach to measure metabolic active cells. To confirm the validity of using Rh123 as a measurement of metabolic activity a comparison was made between stained viable, stained dead, and unstained viable *F. tularensis* cells. The results showed (Fig. 1) that the stained metabolic active cells could be well separated both from stained dead cells and unstained viable cells. Furthermore, almost no difference was found between the two latter cell types (Fig. 1). The background from Rh123-stained dead cells, corresponded to approximately 5×10^3 cells ml^{-1} (Table 1).

3.2. Analyses of total number of F. tularensis cells targeted by monoclonal antibodies

Flow cytometric analysis could also be used for estimation of the total number of cells. However, even if the dilution liquid was filtered three times through 0.22 μm filters, a relatively high background was obtained (data not shown). Consequently, using unstained cells as a measure of total number resulted in a consistent overestimation of the total number of cells. One way to avoid this is to specifically target the cells of interest with antibodies. The monoclonal antibody FT14 directed against the lipopolysaccharide (LPS) of *F. tularensis* [13] was therefore used to specifically identify and quantify the total number of *F. tularensis* cells. Secondary PE-conjugated antibodies were used for flow cytometric detection of bound FT14. The specificity of FT14 was confirmed by incubating FT14 antibodies with *E. coli* and *F. tularensis*, respectively. In similarity to previous results

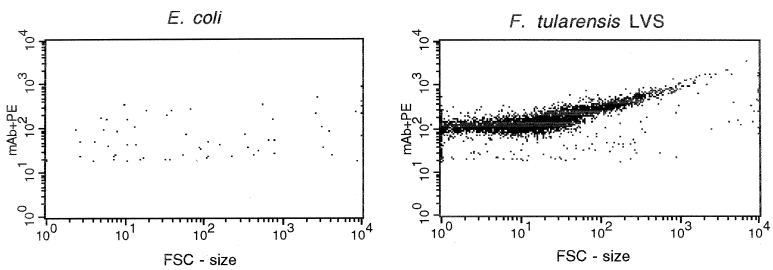

Fig. 2. FC responses for *E. coli* and *F. tularensis* to FT14 monoclonal antibodies.

Table 2
Comparison of precision for different flow cytometric analyses of *F. tularensis* cells

Analysis	Inter-sample variation		Intra-sample variation	
	Mean ± S.D.	CV	Mean ± S.D.	CV
FC_{TOT} (no./ml)	$5.70 \pm 1.20 \times 10^6$	21.1	$9.39 \pm 0.55 \times 10^7$	5.9
MA (no./ml)	$4.79 \pm 1.00 \times 10^6$	20.9	$7.72 \pm 0.46 \times 10^7$	6.0
SR	84.1 ± 3.0	3.5	82.3 ± 0.3	0.35

The results are expressed as coefficient of variation CV (%) between ($n = 10$, 10 samples, 1 analysis/sample) and within ($n = 10$, 1 sample, 10 analyses) samples.

[13], flow cytometric analysis showed no cross-reaction towards *E. coli* (Fig. 2). Next, the flow cytometric method (FC) specific for *F. tularensis* was compared to CC analysis to get information about the obtained yield. The comparison was made on *F. tularensis* cells that had been stored for six weeks in water. The ratio between the total number of *F. tularensis* cells obtained by FC ($6.24 \times 10^{10} \pm 2.14 \times 10^{10}$) and CC ($2.58 \times 10^9 \pm 0.18 \times 10^9$) showed a mean value of 24.2 ($n = 6$). Statistical analysis with T-test showed that the total number determined by the FC was significantly ($P = 0.01$) higher than the values obtained by CC analysis. However, the precision for the FC was lower than for the CC.

3.3. Precision and sensitivity of the method

The precision was investigated for the merged analysis, i.e. staining with Rh123 prior to incubation with monoclonal antibodies and PE-conjugate. Both intra- and inter-sample variations were evaluated. The results showed that the FC-method was highly reproducible (Table 2). The coefficients of variation between samples for total number and metabolic active number were higher than within samples (Table 2). However, the precision expressed as coefficient of variation for SR was below 5%. The sensitivity of the methods was also investigated by different dilutions of an over-night culture of *F. tularensis* (Fig. 3). The sensitivity of the FC method for determining the SR value was about 10^3 cells ml^{-1} under the experimental conditions used.

3.4. Analysis of aerosol samples

To evaluate the method of analysis on aerobiological samples, *F. tularensis* cells were analysed before and after aerosolisation (Table 3). The SR of the samples was analysed by the original methods based on plate count and CC analysis (VC/CC_{TOT}), by the FC-method (MA/FC_{TOT}) and by comparing. All VC/FC_{TOT} methods used for survival analyses showed that SR after aerosol sampling was reduced compared to the original sample used for spraying. The SR based on VC/CC_{TOT}, was about one third compared with the SR based on MA/FC_{TOT}. Calculation of VC/FC_{TOT} showed the lowest decrease in SR after aerosolisation (Table 3).

Table 3
Analyses of samples from three days cultures of *F. tularensis* before and after aerosolisation

Samples	VC (CFU/ml)	CC_{TOT} (no./ml)	MA (no./ml)	FC_{TOT} (no./ml)	SR (VC/CC_{TOT})	SR (MA/FC_{TOT})	SR (VC/FC_{TOT})
	(mean ± S.D.)				(%)		
Generation liquid	$5.30 \times 10^9 \pm 0.74 \times 10^9$	$6.70 \times 10^9 \pm 0.48 \times 10^9$	$7.51 \times 10^{10} \pm 0.35 \times 10^{10}$	$8.12 \times 10^{10} \pm 0.36 \times 10^{10}$	79.1	92.5	6.5
Aerosol sample	$2.15 \times 10^6 \pm 0.48 \times 10^6$	$1.25 \times 10^7 \pm 0.25 \times 10^7$	$2.73 \times 10^7 \pm 0.88 \times 10^7$	$3.74 \times 10^7 \pm 1.08 \times 10^7$	17.2	73.0	5.7

The number of cells in the generation liquid before aerosolisation and the number of cells in the sample collected by the AGI-30 from the aerosol are shown. Values are given as means and S.D. ($n = 3$–6) and compensated (FC) for losses during staining and labelling. The CC values are corrected for the background in the aerosol system given by the generation liquid without bacteria.

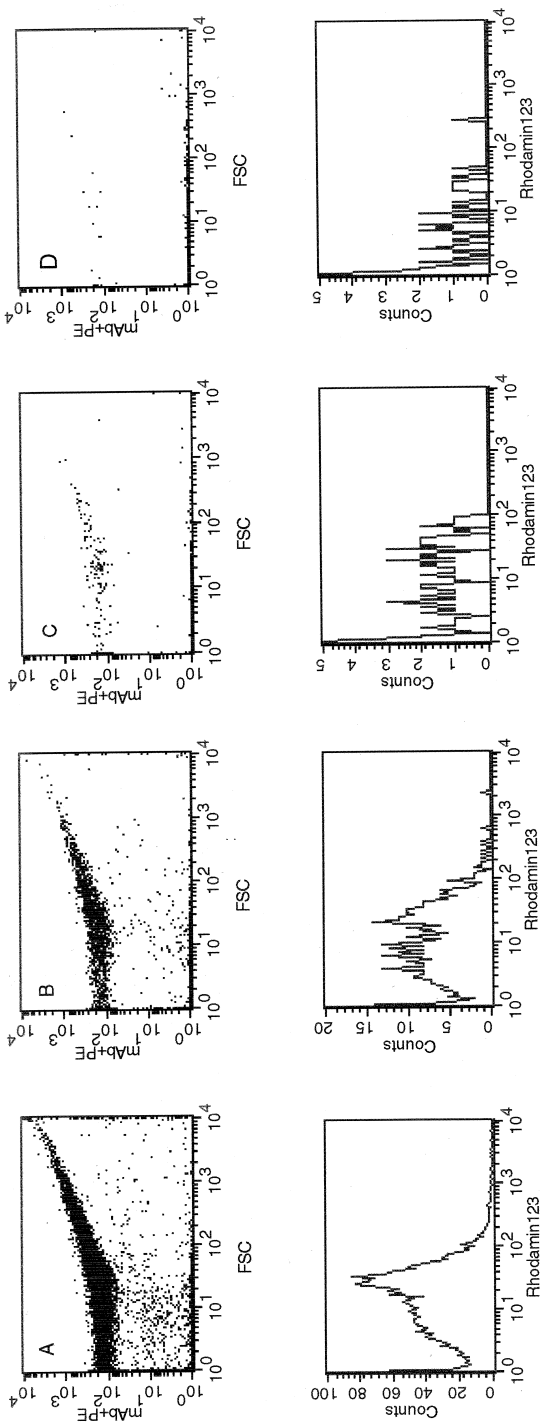

Fig. 3. A typical FC pattern of 10-fold dilutions of viable *F. tularensis* cells. Upper row shows the response for the total number (PE) and the lower row shows the metabolic active number (Rh123) of cells. A–D represents 10-fold dilutions from 10^6–10^3 cells ml^{-1}, respectively. The designation mAb-PE for the *y*-axis in the upper row represents the total number of counts. ←

4. Discussion

F. tularensis is a fastidious bacterium which requires special supplementation of media for its growth, which makes isolation of the bacterium from environment complicated since other bacteria may be favoured on most media utilisable for growth of *F. tularensis* [17]. This is probably one of the reasons why isolates of *F. tularensis* from environmental samples has never, to our knowledge, been reported in Scandinavia, although there are annual reports on sporadic cases or minor epidemics of tularemia from the northern hemisphere in Scandinavia [8,18,19]. There exist, however, other methods for identification of *F. tularensis* that are not dependent on culturing [20–24]. Applications of these methods, without modifications, give, however, little information of the survival ratio of *F. tularensis*. Hence, to learn more about the survival of *F. tularensis* in environmental aerobiological samples improved methods are warranted. This might help to judge the exposure-response relationship. The presented methodological concept for assessment of the survival ratio of *F. tularensis* in liquid aerosol samples is a first step in this context.

The flow cytometric method of analysis was based on the use of monoclonal antibodies and staining with Rh123. The total number of *F. tularensis* cells was determined by specific targeting with monoclonal antibodies and detected by PE-conjugated secondary antibodies. The metabolic active part of the targeted *F. tularensis* cells was quantified by staining with Rh123. The total number of *F. tularensis* cells determined with the FC-method showed higher yield and variation compared to CC analysis. The latter result reinforces previous results where CC-analysis of *E. coli* was found to be the method with the highest precision [5]. The FC-method includes more centrifugation steps, which may contribute to the higher variation. Repeated centrifugation also increases the losses in the FC-method compared to CC-analysis.

This might, however, be minimised by using larger wash volumes in less wash steps and/or PE-conjugated FT14. CC-analysis of *F. tularensis* cells was not optimal since the size of *F. tularensis* is considerably smaller than *E. coli*. Small cells will reduce the yield because the CC cannot resolve bacteria smaller than 0.6 μm. This was also illustrated by the increased discrepancies in yield between FC and CC on starved *F. tularensis* cells compared to aerosolised cells. Other drawbacks with CC are the tedious work with the liquid preparation and that all particles within the selected size-range are counted without discrimination which makes it unsuitable for environmental analyses. In contrast, the FC-method seems suitable for environmental analysis since it was specific for *F. tularensis* and not sensitive to variation in cell size.

The precision in determination of metabolic active cells, by staining with Rh123, was in the same range as for the determination of the total number of cells with antibodies. Even if the coefficient of variation was high for both the denominator and the nominator, the corresponding coefficient for the SR was low. The reason for this was that the same method of analysis was used for total and metabolic number. Values for the denominator and the nominator show covariance that is not always the case when two different methods are used. Hence, the precision of the survival ratio was high. However, the precision of the survival ratio was dependent on the concentration of cells. Below a threshold of approximately 10^3 total number of *F. tularensis* cells, the coefficient of variation in SR ascended (data not shown).

Many investigators have combined the use of discriminative bacterial antibodies and flow cytometric analysis [25–29]. A common problem with antibodies is, however, that they often show a varying degree of cross-reaction with other species. Even when monoclonal antibodies are used, specificity can be a problem for some genera of bacteria [27]. However, the genus *Francisella* is antigenically coherent and the subspecies are indistinguishable by serological methods [30]. Moreover, the monoclonal IgM antibody FT14, directed against the O side chain of the LPS, did not react with LPS samples from 11 different Gram-negative bacteria or 6 species of intact Gram-negative bacteria including *Brucella* [13]. In agreement with this, no cross reaction was observed towards *E. coli* (Fig. 2). Thus, it seems reasonable to assume that the FT14 epitope is unique for the *Francisella* genus. However, serological cross-reaction between *F. tularensis* and *Brucella* species has been reported [31]. The cross-reaction titers were suppressed by dithiothreitol, which indicated cross-reactions due to IgM antibodies [31]. Hence, there might exist potential limitations using IgM antibodies, even if FT14 do not have any known cross-reactivity.

As expected, viable and formalin killed cells could readily be separated in the flow cytometer by the extent to which they accumulated Rh123 (Fig. 1). Comparison of VC yield with MA yield showed consistently higher values for MA. This indicates that VC underestimates the viable microbial concentration. The term 'viable but not culturable' has often been used to refer to cells which display metabolic activity but cannot be cultured [32–34]. Evidence of resuscitation from the viable but not culturable state for human pathogens such as *Vibrio vulnificus* and *Legionella* has been shown to occur [34]. If the result from traditional plate count underestimates the exposure dose from *F. tularensis*, the real infectious dose may be higher than approximated [7]. However, it is not known whether viable but not culturable cells of *F. tularensis* are pathogenic. Still, it could be envisaged, provided that resuscitation is possible under certain circumstances, as yet to be identified, that negative response from plate count does not ensure absence of infection risk.

Bioaerosol samples of *F. tularensis* showed a marked difference in SR based on MA/FC_{TOT} compared with SR based on VC/CC_{TOT}. After aerosolisation mean SR, based on VC/CC_{TOT}, was reduced from 79% to 17%, respectively, whereas the mean SR, based on MA/FC_{TOT}, was reduced from 92% to 73%, respectively (Table 3). The difference in SR for the two methods was less pronounced in the cultures used for generation of the aerosol (Table 3). The lowest reduction in SR (6.5 vs. 5.7%, Table 3) in the aerosol samples was found when SR was calculated from VC/FC_{TOT}. SR based on VC/FC_{TOT} were in the same range as found by Heidelberg et al. [35] directly after aerosolisation of airborne bacteria, as based on acridine orange direct count (AODC) and VC. Interestingly, these authors also showed that culturable cells decreased rapidly with time in aerosol, although the majority of the cells

retained their metabolic activity. However, the time in aerosol state lasted only a few seconds in the experiments reported in this paper. Aerosolisation puts the bacteria under multi-factorial stress due to generation, conditioning and aerosol sampling. One effect of this stress is that the bacteria are reduced in size, which was observed in the analyses by CC and FC in this study (data not shown). Thus, it is reasonable to assume that assessment of SR based on MA/FC_{TOT} better reflected the influence of aerosolisation on *F. tularensis*, since bacterial size alterations and loss of culturability had little effect on the analyses.

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