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Use of flow cytometry for rapid and accurate enumeration of live pathogenic *Leptospira* strains

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ABSTRACT

Enumeration of *Leptospira*, the causative agent of leptospirosis, is arduous mainly because of its slow growth rate. Rapid and reliable tools for numbering leptospires are still lacking. The current standard for *Leptospira* cultures is the count on Petroff-Hausser chamber under dark-field microscopy, but this method remains time-consuming, requires well-trained operators and lacks reproducibility. Here we present the development of a flow-cytometry technique for counting leptospires. We showed that upon addition of fluorescent dyes, necessary to discriminate the bacterial population from debris, several live *Leptospira* strains could be enumerated at different physiologic states. Flow cytometry titers were highly correlated to counts with Petroff-Hausser chambers ($R^2 > 0.99$). Advantages of flow cytometry lie in its rapidity, its reproducibility significantly higher than Petroff-Hausser method and its wide linearity range, from 10^4 to 10^8 leptospires/ml. Therefore, flow cytometry is a fast, reproducible and sensitive tool representing a promising technology to replace current enumeration techniques of *Leptospira* in culture. We were also able to enumerate *Leptospira* in artificially infected urine and blood with a sensitivity limit of 10^5 leptospires/ml and 10^6 leptospires/ml, respectively, demonstrating the feasibility to use flow cytometry as first-line tool for diagnosis or bacterial dissemination studies.

1. Introduction

Leptospirosis is an emerging zoonosis worldwide affecting >1 million humans per year with approximately 60,000 deaths (Costa et al., 2015). The disease is caused by the spirochete *Leptospira*, a spiral-shaped bacterium usually 6–20 μm in length and 0.1 μm in diameter displaying distinct hooked ends (Cameron, 2015). Tools to manipulate this organism are still lacking. In particular, its rapid and reliable enumeration still represents a technical challenge. Classical methods like Colony Forming Units (CFU) counts are not suitable for routine testing considering leptospires slow growth rate. The doubling time of pathogenic species ranges from 6 h to 18 h (Cameron, 2015). 1 to 2 weeks of incubation are required to reach early stationary phase in liquid cultures and >4 weeks to obtain colonies on agar plates (Picardeau, 2015). The current standard method for enumerating *Leptospira* in cultures is the count of motile bacteria on a Petroff-Hausser chamber under dark-field microscopy. Due to *Leptospira* high motility within or between microscope fields, this method remains time-consuming, requires a well-trained operator and lacks reproducibility (Murray et al., 2010). Efforts have been made to develop other enumeration techniques. The optical density determination is fast but does not quantify viability. In addition, *Leptospira* show low absorbance properties (Schreier et al., 2009) and as a consequence, at least 10^7 to 10^8 bacteria/ml are required for a detectable OD with a maximum of 1 for stationary phase cultures. This technique has therefore limited sensitivity and precision. Other methods like measurement of electric resistance using a Coulter counter (Humberd et al., 2005) or quantitative real-time PCR based on DNA quantification (Lambert et al., 2012; Lourdault et al., 2009) were also used on *Leptospira* but do not allow to discriminate live from dead leptospires. Bioluminescence approaches have been developed in 1986 by Nervig and coworkers using an ATP assay, which was labor-intensive and valid only in the limited range of 4×10^8 to 8×10^9 leptospires/ml (Nervig et al., 1986). More recently, Murray and colleagues modified Manilae and Patoc strains by inserting the *luxCDABE* cassette in their genomes, resulting in an efficient counting method but restricted to genetically modified strains (Murray et al., 2010). Therefore, there is still a lack of a fast and sensitive method for enumeration of live leptospires.

In the past years, flow cytometry (FCM) has been increasingly used in diverse fields of microbiology like medicine (Karo et al., 2008; Kempf et al., 2005; Saito et al., 2005; Schmidt et al., 2006; Shrestha et al., 2011, 2012), environmental studies (Casamayor et al., 2007; De Roy et al., 2012; Füchslin et al., 2010; Taguri et al., 2011) and food industry (Comas-Riu and Rius, 2009; Gunasekera et al., 2000). In 2003, FCM was applied to leptospires as a serological diagnosis tool to quantify

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aggregation of bacteria in contact with patient sera, but not to characterize and count these bacteria at a single-cell level (Yitzhaki et al., 2004). FCM was also used to efficiently enumerate the spirochetes *Borrelia burgdorferi* (Bakker et al., 2007) and *Treponema denticola* (Orth et al., 2010). Here, we describe a fast, reproducible and sensitive flow cytometry method to enumerate live *Leptospira* in culture. We also demonstrate the feasibility to use flow cytometry as first-line tool for diagnosis or bacterial dissemination studies.

2. Material and methods

2.1. Bacterial strains and culture conditions

Leptospira interrogans serogroups Australis, Canicola and Icterohaemorrhagiae were grown in Ellinghausen-McCullough-Johnson-Harris medium (EMJH) at 29 °C and 80 rpm. Growth kinetics of the Australis strain was followed after inoculation of 1 ml of a mid-log phase culture into 50 ml of fresh EMJH medium, and enumerated during 9 days using Petroff-Hausser and flow cytometry methods (see below). Canicola and Icterohaemorrhagiae strains were enumerated at 3 points corresponding to the start of log phase, mid-log phase and stationary phase. When needed, the bacteria were diluted in saline solution (Gibco).

Killed cultures were obtained by heating at 70 °C during 10 min. A mix of fresh and killed bacteria was obtained by mixing 500 μl of the fresh culture with 500 μl of the killed culture.

For analyses of leptospires in biological samples, sterile blood and urine samples were collected from three healthy dogs (Merial, France). Blood samples were stored in heparin coated tubes. 50 μl of pure or diluted leptospires from Australis serogroup were spiked in triplicate in 450 μl of urine or blood. When indicated, these artificially infected urine and blood preparations were diluted in saline solution.

2.2. Enumeration of Leptospira using Petroff-Hausser counting chamber

Live motile leptospires were enumerated on a Petroff-Hausser counting chamber under a dark-field microscope with a ×40 objective (Eclipse Ni, Nikon). Appropriate dilutions were performed in saline in triplicate. Each suspension was loaded into a counting chamber, and each chamber was analyzed by 2 operators. Enumerations were performed right after slides preparation to prevent adhesion or movement of the bacteria to the borders of the slides. Counts were performed over the 5 large squares in the diagonal of the chamber, containing 16 small squares each.

2.3. Staining of Leptospira

Leptospires suspensions were stained with two dyes from the LIVE/DEAD® BacLight™ Bacterial Viability kit (L7012, Life Technologies) added at a 1:1000 dilution each: SYTO® 9, a green-fluorescent nucleic acid dye staining all cells, and Propidium Iodide (PI), a high-affinity red-fluorescent nucleic acid dye staining only cells with altered-membranes. Maximal wavelengths of excitation and emission are respectively at 485 nm and 498 nm for SYTO® 9, and 535 nm and 617 nm for PI. Leptospires were shortly vortexed after dyes addition, incubated 3 min in the dark and vortexed a second time just before analysis by flow cytometry.

2.4. Analysis of Leptospira using flow cytometry

Analyses were performed on the BD Accuri™ C6 (BD Biosciences). This benchtop and ready-to-use flow cytometer is composed of a blue laser emitting at 488 nm, a forward scatter detector (FSC) at 0°, a side scatter detector (SSC) at 90°, a green fluorescence detector at 533 ± 15 nm and a red one >670 nm. No voltage adjustments are required. An automatic calculation allows the determination of the absolute count of events per microliter. Settings were adjusted as follows: speed of analysis at 14 μl/min; runs limited either in time (35 s for feasibility assays and 90 s for linearity studies in EMJH, urine and blood), or in number of events (20,000 for viability and correlation with Petroff-Hausser studies). Data were acquired *via* the BD Accuri™ C6 software and collected on 2 plots in logarithmic scales: SSC *versus* FSC, or red fluorescence channel (renamed PI for clarity, corresponding to red fluorescence) *versus* green fluorescence channel (renamed SYTO® 9 for clarity, corresponding to green fluorescence). Thresholds of acquisition were applied either on FSC parameter (1000, arbitrary units) or on green fluorescence channel parameter (2000 or 12,000, arbitrary units) as indicated. Gates containing leptospires were drawn on FSC/SSC and SYTO® 9/PI plots. For urine analyses, the display on SYTO® 9/PI plots was restricted to the events belonging to the gate drawn on the FSC/SSC plot. For blood analyses, the acquisition was restricted to the events belonging to this FSC/SSC gate.

2.5. Statistical analyses

Statistical analyses were performed using Statgraphics Centurion XV software, version 15.2.14 (Statpoint Technologies, VA, USA). All tests were performed with a standard 5% α-risk. Correlation of FCM with Petroff-Hausser method and linearity of enumerations in EMJH, urine and blood were analyzed using linear regressions. The linearity range was determined using a lack of fit test (non-significant lack-of-fit was required). The proportionality of the method and the correlations between methods were validated if: (i) value 1 was included in the 95% confidence interval for the slope and (ii) the intercept was not significantly different from zero. Precision of FCM and Petroff-Hausser techniques was assessed by a multifactor analysis of variances (MANOVA). The precision variances of both techniques were calculated as the sum of the operator and the repeatability variance components. The variances of the two techniques were compared to each other using an F-test.

3. Results

3.1. Feasibility of Leptospira detection by flow cytometry

To determine the possibility of distinguishing leptospires from background signals with the BD Accuri™ C6 flow cytometer, we compared the dot plot of a dense culture diluted at 1:1000 to those of control samples containing saline or EMJH. We observed a dense population of events with low FSC and SSC parameters in the controls, corresponding to the background signal caused by the particles of the medium and electronic noise (Fig. 1A). For the bacterial culture, FSC was identical to the controls while SSC was only slightly higher (Fig. 1B), indicating that leptospires cannot be efficiently separated from the background on FSC/SSC parameters.

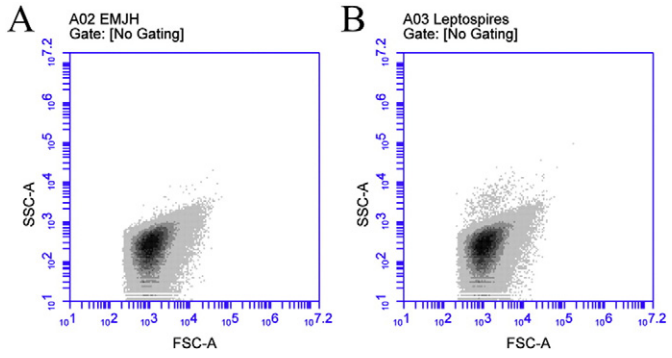

Fig. 1. Detection of *Leptospira* by flow cytometry. FSC/SSC dot plots of (A) EMJH and (B) a culture of leptospires. A low threshold was applied at 1000 on FSC parameter. The dot plot of saline solution was identical to EMJH (data not shown).