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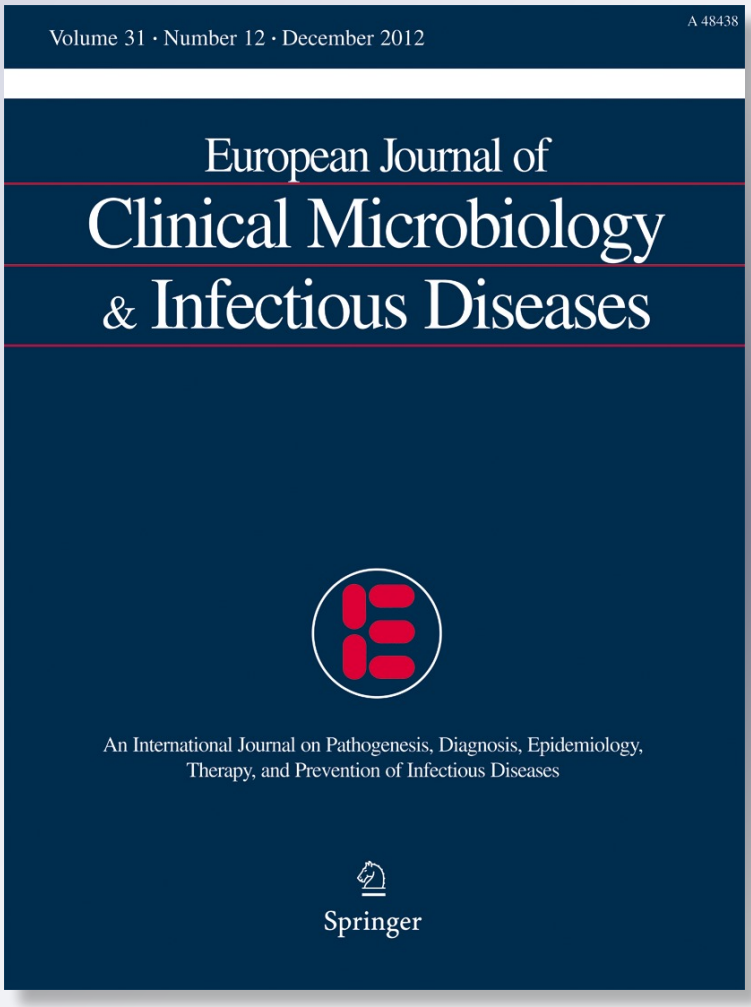

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ARTICLE

Detection of *Legionella pneumophila* on clinical samples and susceptibility assessment by flow cytometry

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Abstract Culture in selective media represents the standard diagnostic method to confirm *Legionella pneumophila* infection, despite requiring a prolonged incubation period; antigen detection by immunofluorescence (IFS) and molecular techniques are also available, but they do not allow antimicrobial susceptibility evaluation. Our objective was to optimise flow cytometry (FC) protocols for the detection of *L. pneumophila* in respiratory samples and for susceptibility evaluation to first-line drugs. In order to optimise the FC protocol, a specific monoclonal antibody, conjugated with fluorescein isothiocyanate (FITC), was incubated with type strain *L. pneumophila* ATCC 33152. The limit of detection was established by analysing serial dilutions of bacterial suspension; specificity was assayed using mixtures of prokaryotic and eukaryotic microorganisms. The optimised FC protocol was used to assess 50 respiratory samples and compared with IFS evaluation. The susceptibility profile to erythromycin, ciprofloxacin and levofloxacin was evaluated by FC using propidium iodide and SYBR Green fluorescent dyes; the results were compared with the Etest afterwards. The optimal specific antibody concentration was 20 μg/ml; 10^2/ml *Legionella* organisms were detected by this protocol and no cross-reactions with other microorganisms were detected. The five positive respiratory samples (10 %) determined by IFS were also detected by FC, showing 100 % correlation. After 1 h of incubation at 37 °C with different antimicrobials, SYBR Green staining could discriminate between treated and non-treated cells. A novel flow cytometric approach for the detection of *L. pneumophila* from clinical samples and susceptibility evaluation is now available, representing an important step forward for the diagnosis of this very relevant agent.

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Introduction

Legionella pneumophila is responsible for Legionnaires' disease, a severe atypical pneumonia [1, 2], but also for Pontiac fever, a rare and self-limiting non-pneumonic illness [3]. *L. pneumophila* incidence is increasing in the United States and other countries [4]. In Europe, the overall incidence in 2008 was estimated to be around 11.8 cases per million [5] and the figure is still increasing [1].

The genus *Legionella* encompasses 50 species and 72 serogroups, with serogroup 1 being responsible for almost 90 % of all reported legionellosis cases [1]. *L. pneumophila* infection usually occurs through the inhalation of contaminated aerosols, highlighting the risks inherent to the presence of such organisms in wet cooling systems or water distribution systems

in healthcare facilities [6]. Several host risk factors have recently been described in both healthcare-related and community-acquired infections caused by *L. pneumophila*, such as immunosuppression, cancer, organ transplantation, corticosteroid therapy and treatment with tumour necrosis factor-α antagonists [1]. Legionnaires' disease is associated to a high mortality rate (15–20 %); it involves inconsistent and non-specific clinical manifestations, thus, representing a challenging diagnosis [1]. Culture in selective media is considered to be the standard approach for the definitive laboratorial diagnosis of *L. pneumophila*, despite the prolonged incubation period. Direct fluorescence antibody detection in respiratory secretions and tissue samples is an alternative methodology used routinely. Despite some advantages when compared to culture, it requires an expert and well-trained observer. In addition, it can cross-react with several other bacterial agents (like *Bacteroides fragilis*, *Pseudomonas* spp., *Stenotrophomonas* spp. and *Flavobacterium* spp.), resulting in low specificity [7]. Polymerase chain reaction (PCR) is highly sensitive and specific; however, it is often unavailable in some clinical laboratories and also gives no information regarding *Legionella* antimicrobial susceptibility evaluation. Antimicrobial susceptibility profile evaluation, which is not routinely performed, can only be assessed using cultural methods, which are time-consuming and provide late results [8–10]. Flow cytometry (FC) is an established cell analysis technology which provides a quantitative and qualitative multi-parametric cell analysis based upon light-scattering and fluorescent signals [11]. This tool is still somewhat underestimated in microbiology but yields a very high potential. Our research group has described several applications regarding the detection of several microorganisms [12–16], as well as susceptibility evaluation of bacteria [17], fungi [18, 19] and parasites [12]. FC has previously been used for the detection of *Legionella* in environmental specimens and experimental systems [8, 20]; however, it has been rarely applied to detect *L. pneumophila* in clinical specimens [21].

Materials and methods

Bacterial strains

L. pneumophila type strain 33152 from the American Type Culture Collection (ATCC) was used for protocol optimisation and susceptibility studies. This strain was grown in buffered charcoal yeast extract (BCYE, Oxoid, London, England) and incomplete BCYE without L-cysteine and ferric pyrophosphate for a period of 3–5 days at 35±1 °C with an atmosphere of 3–5 % carbon dioxide. *Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 90028 type strains were used for specificity studies of the staining.

Optimisation of the staining procedure

Suspensions of *L. pneumophila* ATCC 33152 were adjusted to a density of 0.5 McFarland (10^8 bacterial cells/ml), turbidity standard, in 0.9 % NaCl solution. For safety reasons, whenever possible, suspensions were heated at 100 °C for 20 min and death was confirmed by culture. Different FC staining protocols were optimised, namely: (a) for *Legionella* spp. detection, dead cells were incubated with serial concentrations (10, 20, 30 and 40 μg/ml) of the monoclonal antibody MONOFLUO™ Anti-*Legionella pneumophila* (Bio-Rad, Hercules, CA, USA) conjugated with fluorescein isothiocyanate (FITC). This antibody is specific and reacts with an external membrane protein present in all known *L. pneumophila* serogroups; (b) for susceptibility evaluation, propidium iodide (PI, 1 μg/ml) was added to both dead and viable bacterial suspensions (10^8 cells/ml) during 30 min at 37 °C, protected from light; SYBR Green I (1 μg/ml), a fluorescent dye that binds to double-stranded DNA was added only to viable cells during 1 to 4 h, at 37 °C.

After incubation with the tested dyes, the bacterial suspensions were centrifuged at 3,000 × *g* for 10 min and the supernatant was discharged; the pellet was re-suspended in 1 ml of phosphate-buffered saline (PBS), transferred to a propylene tube and analysed by FC.

Flow cytometry analysis

The characteristics of *L. pneumophila* cells were evaluated in a FACSCalibur (BD Biosciences, Sydney, Australia) flow cytometer equipped with three PMT standard filters (FL1: BP 530/30 nm; FL2: BP 585/42 nm; FL3: LP 650 nm), one Argon laser of intensity 15 mW and wavelength 488 nm, and Cell Quest Pro software (version 4.0.2, BD Biosciences Sydney, Australia). Operating conditions included log scales on all detectors [forward scatter (FSC), side scatter (SSC) and fluorescence detectors (FL1)]. Acquisition settings were defined using a non-stained sample (autofluorescence) and adjusting the PMTs' voltage to the first logarithmic (log) decade. The cytometric protocol was set concerning the size and complexity of the analysed particles, as well the intensity of the fluorescent probes used. Reports regarding the fluorescence intensity in FL1 and FL3, and gates corresponding to *L. pneumophila* cells were defined. Instrument controls followed the standard procedures and the instrument settings defined were as follows: FSC E01, SSC 474 V,

fluorescence detectors FL1 609 V and FL3 564 V, and SSC threshold 488 V.

Assessment of the detection limit and specificity of the staining protocol

The detection limit of the procedure was investigated using *L. pneumophila* ATCC 33152 serial suspensions in sterile PBS (10^4, 10^3, 10^2 and 10 bacterial cells/ml), which were stained with the MONOFLUO™ Anti-*Legionella pneumophila* (Bio-Rad, Hercules, CA, USA) conjugated with FITC, at 37 °C during 30 min, according to the manufacturer's instructions. After incubation, samples were centrifuged at 3,000 × *g* for 10 min; the supernatant was discarded and the pellet re-suspended in 1 ml of sterile H_2O, vortexed for 30 s, transferred to a propylene tube and analysed by FC. For specificity assessment, 10^6 cells/ml of different microbial suspensions, namely, of *E. coli* type strain ATCC 35218, *S. aureus* ATCC 25923 and *C. albicans* ATCC 90028, were stained with the specific *L. pneumophila* antibody according to the previously optimised condition; afterwards, the suspensions were analysed by FC either in the presence or in the absence of *L. pneumophila*.

Respiratory samples

Fifty respiratory samples from putative Legionnaires' disease patients, obtained from distinct Portuguese hospitals, were frozen at −20 °C within 48 h and analysed in this study:

1. Immunofluorescence staining: 20 μl of each sample were placed on a glass slide and air-dried, followed by fixation. Afterwards, MONOFLUO™ Anti-*Legionella pneumophila* (Bio-Rad, Hercules, CA, USA) conjugated with FITC staining was performed according to the manufacturer's instructions and analysed under immunofluorescence (IFS) microscopy. Samples were considered to be positive for *L. pneumophila* whenever green short bacilli were visualised, either isolated or in a cluster.
2. Flow cytometric analysis: samples were thawed and treated with N-acetyl-L-cysteine (Merck®) for 20 min at 37 °C for complete mucus dissolution and filtered (using a 30-μm pore diameter, Partec CellTrics®). Following centrifugation at 3,000 × *g* for 5 min, 20 μl of the antibody (at the previously optimised concentration) were added to 100 μl of pellet. A set of samples was stained without the previous filtration step. All clinical samples were analysed by both IFS and FC according to the previously described conditions, and the results were subsequently compared. All analyses were performed at least in duplicate.

Antimicrobial susceptibility testing

Following all the biosafety standards and procedures recommended, *L pneumophila* ATCC 33152 was cultivated in solid Mueller–Hinton (MH) medium (Liofilchem®) supplemented with 0.04 %L-cysteine and 0.1 % of ferric phosphate during 5 days at 37 °C. The Etest (AB bioMérieux, Solna, Sweden) was performed (24 h of incubation at 37 °C) on the antimicrobials under study, namely, erythromycin, ciprofloxacin and levofloxacin (Sigma-Aldrich®). For FC susceptibility assessment, following 18 h of incubation at 37 °C in MH broth medium (Liofilchem®) supplemented with 0.04 % L-cysteine and 0.1 % of ferric phosphate, suspensions of *L. pneumophila* ATCC 33152 were adjusted to a density of 0.5 McFarland (10^8 bacterial cells/ml). The bacterial suspensions were afterwards adjusted with fresh supplemented MH broth medium to 10^6 bacterial cells/ml and incubated from 1 h to 4 h at 37 °C with each of the studied antimicrobials, at 1 and 10 μg/ml. Consequently, the suspensions were centrifuged at 3,000 × *g* for 10 min and the supernatant discharged; the pellet was re-suspended in 1 ml of PBS and transferred to a propylene tube; two different fluorescent probes, PI or SYBR Green, were used to stain the cells and analysed by FC according to the previously optimised conditions.

Results

Legionella detection

Optimisation of the FC protocol

During the optimisation studies aiming to determine the specific antibody concentration, as we increased its concentration, an increase of the mean intensity of fluorescence (MIF) was evident. Twenty microlitres per millilitre of specific antibody yielded the highest value of MIF at FL1 (530/30 nm), being similar to 30.0 or 40.0 μg/ml (data not shown); thus, 20 μg/ml was the concentration used in further experiments.

Sensitivity and specificity of the FC method

Concerning the detection limit of the cytometric method, 10^2 *Legionella* cells/ml was the lowest concentration that could be detected, since distinction of *L. pneumophila* cells was not possible in higher dilutions. Regarding the specificity of the staining, microorganisms that are usually present in respiratory samples were mixed with negative and positive control samples, and subsequently incubated with the specific monoclonal antibody. Although an increase in the number of events was detectable, specific staining could

only be detected in suspensions containing *L. pneumophila* organisms, as represented in the R region (Fig. 1).

Respiratory samples

Following IFS, 5 samples (10 %) were positive for *L. pneumophila*. Sample preparation included treatment with a mucolytic agent, with and without filtration (30-μm pore size). A higher number of non-fluorescent cytometric events was detected in samples without filtration and a better separation of stained bacterial cells from debris was obtained with filtration without loss of signal (data not shown). For FC analysis, we considered to be positive all the respiratory samples displaying events at the R acquisition gate corresponding to *L. pneumophila* cells stained with specific antibody (typical example shown in Fig. 2); a clear distinction between positive and negative clinical samples was found. Considering IFS, 100 % correlation with FC was found.

Legionella antimicrobial susceptibility testing

Etest results

Regarding the susceptibility studies involving the ATCC strain, the minimum inhibitory concentration (MIC) values of ciprofloxacin, levofloxacin and erythromycin by the Etest were 0.03, 0.03 and 0.25 μg/ml, respectively, which are in agreement with the expected results.

For the flow cytometric results, PI was able to stain all the dead bacterial population (heat-treated) but not the drug-treated cells, even with the highest concentration for 4 h. However, after SYBR Green staining, non-treated cells showed two populations, corresponding to different amounts of DNA (n and 2n; Fig. 3a). After incubation with different antimicrobials, three populations with different intensities of fluorescence could be seen, corresponding to a third population displaying a higher intensity of fluorescence, i.e. higher DNA amount (Fig. 3b–d). For all the tested antimicrobials, as well as to the different tested concentrations, similar results were found (data not shown).

Discussion

Specific and fast diagnosis of *L. pneumophila* infection has a significant clinical relevance [1]. We report on the development of a flow cytometric protocol for *L. pneumophila* detection and its validation through the testing of respiratory samples. For safety purposes, the majority of optimisation procedures were performed with dead microorganisms, given that *L. pneumophila* aerosols constitute a threat to

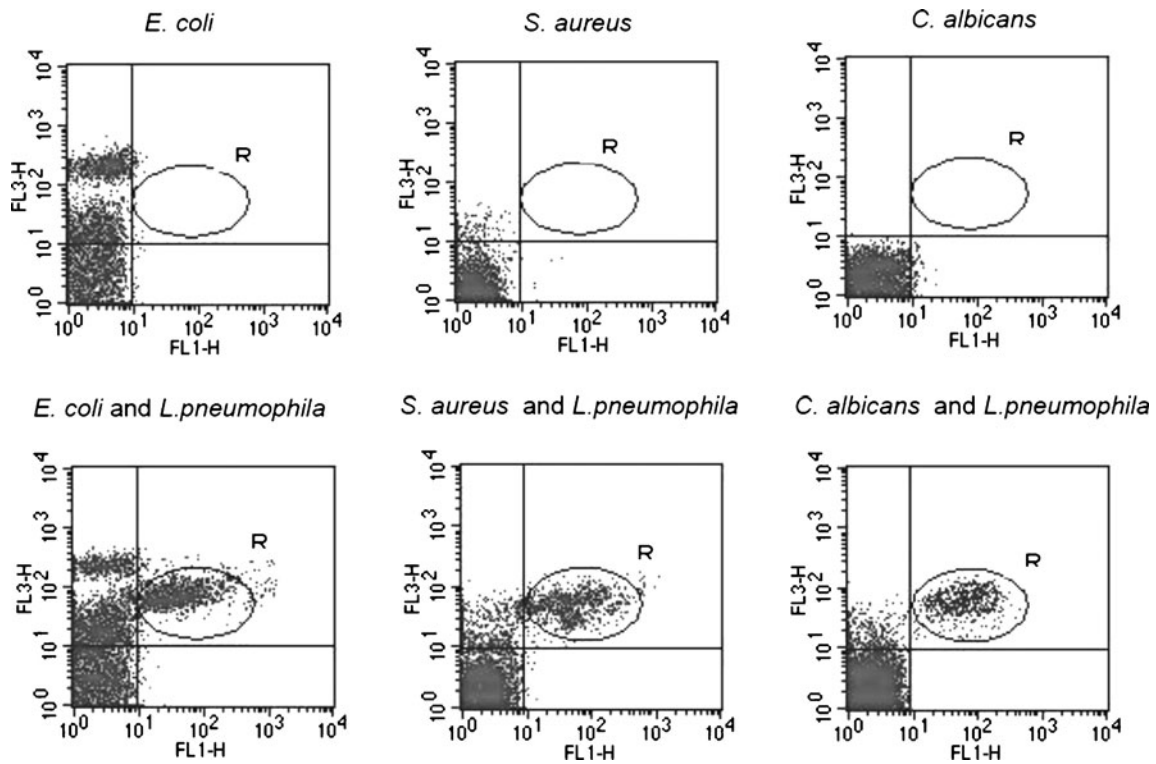

Fig. 1 Two-dimensional dot plot correlating FL1 (green fluorescence, 535 nm) with FL3 (red fluorescence, 620 nm) of different pure bacterial suspensions: **a** *Escherichia coli*, **b** *Staphylococcus aureus* and **c** *Candida albicans* isolated and associated with *Legionella pneumophila* after staining with specific monoclonal antibody MONOFLUO™ Anti-*Legionella pneumophila*. Region R corresponds to the *L. pneumophila* acquisition gate

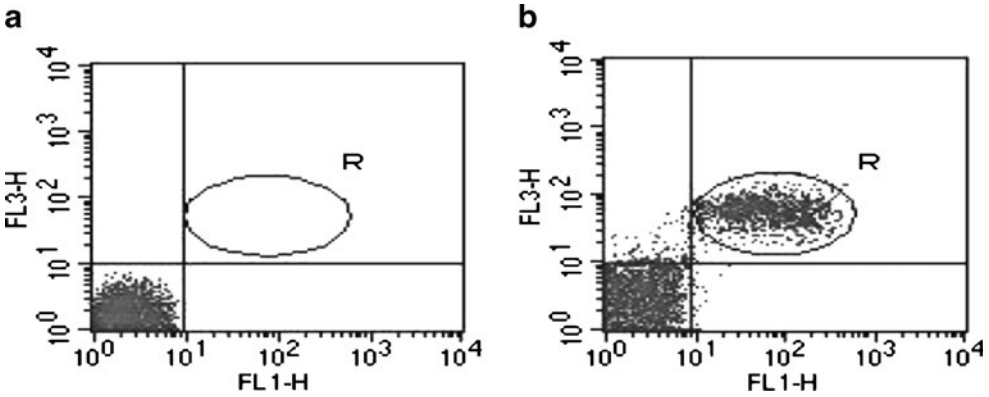

Fig. 2 Two-dimensional dot plot correlating FL1 (green fluorescence, 535 nm) and FL3 (red fluorescence, 620 nm) of: **a** negative sample without *L. pneumophila* cells and **b** 10^4 bacteria/ml *L. pneumophila* ATCC 33152 cell suspension labelled with 20 μl of specific monoclonal antibody MONOFLUO™ Anti-*Legionella pneumophila* conjugated with fluorescein isothiocyanate (FITC) and 1 μg/ml of propidium iodide (PI). Region R corresponds to the specific acquisition gate for labelled *L. pneumophila* cells

laboratory personnel. Considering the increasing complexity of diagnostic laboratory techniques, resulting in higher costs and the need for high technical expertise, the development of more sensitive and automated methods will allow the rapid assessment of clinical specimens, which is in high demand. FC is a method based upon the evaluation of cell fluorescence which has frequently been shown to be specific and yielding high sensitivity. As mentioned previously, the monoclonal antibody used for FC analysis and IFS assay is specific for *L. pneumophila*. We developed and optimised a cytometric analytical protocol for respiratory clinical samples. For this purpose, an important condition should be fulfilled: the concentration of the probe should be at its minimum to allow a clear separation of the stained population from other events; while insufficient staining would not allow a clear separation from debris, excessive staining could result in unspecific fluorescence. Since cytometric analysis could be difficult to perform in the presence of organic debris, the filtration step prior to antibody staining resulted in improved discrimination of *L. pneumophila* organisms. In order to confirm the specificity of the monoclonal antibody, cross-reactions were investigated using polymicrobial suspensions. Prokaryotic (*E. coli*, *S. aureus*) and eukaryotic microorganisms (*C. albicans*) were mixed with *L. pneumophila*. An increased number of events were detected, although without staining with the specific antibody, demonstrating that the reagent is quite specific (Fig. 1).

The detection limit of the assay has relevant clinical implications, despite the number of organisms needed to cause infection in humans remaining undetermined [21]. FC usually allows the detection of low threshold limits, which are difficult to achieve by standard methods [17–19, 21]. A few previous studies had previously described the application of FC to *L. pneumophila* detection, both in water and respiratory samples, but few indicated the threshold limit [20, 21]. In this study, we were able to define clearly a threshold as low as 10^2 bacterial cells/ml.

Susceptibility testing of *L. pneumophila* is not usually performed, since it is a cumbersome procedure and this organism remains susceptible to antibiotic drugs commonly used for treatment. However, susceptibility trends of these pathogens should be monitored periodically in both clinical and environmental isolates. Some authors have described

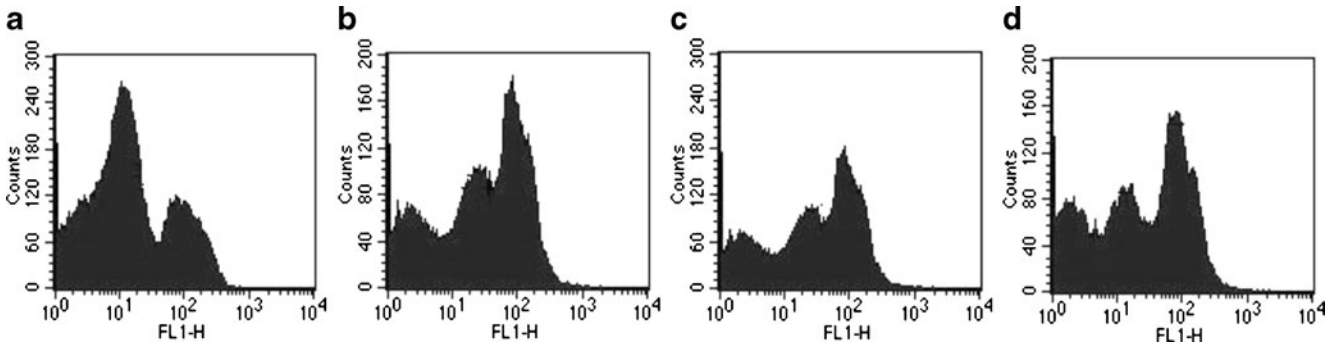

Fig. 3 Histograms correlating counts versus fluorescence intensity of *L. pneumophila* marker (FL1: green fluorescence, 535 nm) of 10^4 bacteria/ml *L. pneumophila* ATCC 33152 cell suspension labelled with 1 μg/ml of SYBR Green I: **a** non-treated cell suspensions and cell suspensions treated with 1 μg/ml of antimicrobials, namely: **b** ciprofloxacin, **c** erythromycin and **d** levofloxacin

the resistance mechanisms of *L. pneumophila* against fluoroquinolones, a class of antibiotics commonly prescribed [22, 23], which is an alert to the illusory idea that it is very rare to find resistance among such pathogens. However, in the last few years, no new reports have been made and, also, no reports on clinical failure associated with increased MIC or the detection of resistance mechanisms have been published. Therefore, surveillance studies of the in vitro activities of antibiotics used for Legionnaires' disease are necessary in order to identify changes in susceptibility profiles.

A standard method for the assessment of susceptibility profiles to antibiotics is not available for *Legionella* spp. [24]. Previous studies concerning *Legionella* susceptibility testing in vitro used broth dilution, cell culture testing and Etest evaluation; however, all these tests require growth during a long period of time (48 to 72 h) [25–27].

Despite the in vivo evidence of treatment failure in patients with Legionnaires' disease receiving such antibiotics being scarce [28], the development and implementation of easier and faster alternatives for the susceptibility testing of *L. pneumophila* in order to monitor the possible emergence of resistant isolates is of relevance. The susceptibility test hereby described based on FC will allow patients presenting atypical pneumonia to be prescribed a targeted therapy, which can potentially lead to improved clinical outcome Legionnaires' disease.

The studied fluoroquinolones, ciprofloxacin and levofloxacin, inhibit the DNA gyrase, while erythromycin is macrolide targeting the ribosomal machinery, meaning that both classes of antibiotics lead to the inhibition of the bacterial DNA replication. Therefore, it was expectable that PI did not stain the cells, since no membrane lesion occurs according to the mode of action of these drugs. On the other hand, SYBR Green was showed to be a suitable fluorochrome because it is able to diffuse across the cellular membrane, staining double-stranded DNA, thus, unveiling the normal or an impaired cell cycle. The amount of DNA of viable cells, without any antibiotic treatment, showed a normal cell cycle pattern—two populations were found, namely, n and 2n. The antibiotic stress induced a third population, with an increased abnormal DNA content (*ploidy*) as expected, since replication is blocked by the antibiotics [29]. A new cytometric detection method is now available, providing a more reliable detection of *L. pneumophila* organisms in respiratory clinical samples and fast assessment of its susceptibility profile.

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Conflict of interest The authors declare that there is no source of funding and no potential conflicts of interest.

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