Journal of Microbiological Methods 109 (2015) 123–128

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Isolation of single *Chlamydia*-infected cells using laser microdissection

Oleg V. Podgorny [a,b,*], Nadezhda F. Polina [a], Vladislav V. Babenko [a], Irina Y. Karpova [a], Elena S. Kostryukova [a], Vadim M. Govorun [a,c,d], Vassili N. Lazarev [a,d]

[a] Scientific Research Institute of Physico-Chemical Medicine, Malaya Pirogovskaya Str. 1a, Moscow 119435, Russia
[b] Koltzov Institute of Developmental Biology of the Russian Academy of Sciences, Vavilov Str. 26, Moscow 119334, Russia
[c] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Miklukho-Maklaya Str., 16/10, Moscow 117997, Russia
[d] Moscow Institute of Physics and Technology (State University), Institutskiy per. 9, Dolgoprudny, Moscow Region 141700, Russia

ARTICLE INFO

Article history:
Received 1 July 2014
Received in revised form 12 December 2014
Accepted 23 December 2014
Available online 26 December 2014

Keywords:
Laser microdissection
Chlamydia
Whole genome sequencing

ABSTRACT

Chlamydia are obligate intracellular parasites of humans and animals that cause a wide range of acute and chronic infections. To elucidate the genetic basis of chlamydial parasitism, several approaches for making genetic modifications to *Chlamydia* have recently been reported. However, the lack of the available methods for the fast and effective selection of genetically modified bacteria restricts the application of genetic tools. We suggest the use of laser microdissection to isolate of single live *Chlamydia*-infected cells for the re-cultivation and whole-genome sequencing of single inclusion-derived *Chlamydia*. To visualise individual infected cells, we made use of the vital labelling of inclusions with the fluorescent Golgi-specific dye BODIPY® FL C5-ceramide. We demonstrated that single *Chlamydia*-infected cells isolated by laser microdissection and placed onto a host cell monolayer resulted in new cycles of infection. We also demonstrated the successful use of whole-genome sequencing to study the genomic variability of *Chlamydia* derived from a single inclusion. Our work provides the first evidence of the successful use of laser microdissection for the isolation of single live *Chlamydia*-infected cells, thus demonstrating that this method can help overcome the barriers to the fast and effective selection of *Chlamydia*.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Chlamydia are a large group of Gram-negative obligate intracellular bacteria that cause a broad range of diseases in a variety of species, including humans. *Chlamydia* are characterised by a unique biphasic life cycle lasting 48–72 h that is represented by an alternation between two functionally and morphologically distinct forms of microorganisms that are well-adapted for extracellular survival and intracellular multiplication (reviewed in (Abdelrahman and Belland, 2005; Bastidas et al., 2013)). Infection begins when a spore-like, metabolically non-active form of the bacteria termed elementary bodies (EBs) attach to and are internalised into a host cell using an unknown mechanism resembling endocytosis (Hybiske and Stephens, 2007a). Following internalisation, EBs rapidly modify vesicles using *Chlamydia*-derived proteins to fuse and form single membrane-bound parasitophorous vacuoles within cell, termed inclusions (Hackstadt et al., 1999), and to avoid the endocytic pathway (reviewed in (Fields and Hackstadt, 2002)). Within the inclusion, the EBs differentiate into metabolically active and replicative form of the bacteria termed reticulate bodies (RBs). RBs undergo repeated cycles of binary fission. RBs begin to differentiate asynchronously back into the EB form in the middle of the infectious cycle (reviewed in (Abdelrahman and Belland, 2005; Bastidas et al., 2013)). A new generation of EBs is released through cell lysis or extrusion to infect neighbouring cells, thus initiating a new round of infection (Hybiske and Stephens, 2007b).

To understand microbial virulence and antibiotic resistance and hence to develop effective therapies and prevent the spread of *Chlamydia* infection, we must investigate the genetic basis of extracellular survival, intracellular multiplication, tissue tropism and the niche-specific adaptation of bacteria. Whole genome sequencing and several successful examples of the transformation and mutagenesis of *Chlamydia*, which have been recently published, suggest that the era of using genetic tools to investigate *Chlamydia* has begun (Harris et al., 2012; Kari et al., 2011; Nguyen and Valdivia, 2012; Wang et al., 2011; Ding et al., 2013; Agaisse and Derré, 2013). Due to its obligate intracellular lifestyle, one of the main limitations to the use of genetic tools in *Chlamydia* is the lack of methods for the fast and effective selection and cloning of transformants or mutants. Several specific techniques have been discussed for obtaining microbiological clones of *Chlamydia*. These include plaque cloning (Banks et al., 1970; Matsumoto et al., 1998), focus cloning (Gieffers et al., 2002) and the clonal isolation of infected cells using fluorescence-activated cell sorting (Alzhanov et al., 2007). The first technique is an inexpensive but time-consuming procedure. This technique is not universal because some *Chlamydia* species and strains (for example, *Chlamydia pneumoniae* strain AR-39 (Gieffers et al., 2002)) do not form plaques in the host cell monolayer. The second

* Corresponding author at: Scientific Research Institute of Physico-Chemical Medicine, Malaya Pirogovskaya Str. 1a, Moscow 119435, Russia.
E-mail address: olegpodgorny@inbox.ru (O.V. Podgorny).

http://dx.doi.org/10.1016/j.mimet.2014.12.018
0167-7012/© 2014 Elsevier B.V. All rights reserved.

technique was developed to resolve this problem, but it involves picking up an individual focus (clone), which has the potential risk that the EBs picked from one focus could be contaminated by the EBs from neighbouring foci. The last technique is a rapid method for clone generation, but it results in significant cell death and false positive sorting because it is based on the discrimination of fluorescence intensity only.

Recently, a protocol for the isolation of single live cells by laser microdissection with gravity transfer (LMDGT) was developed (Podgorny, 2013). LMDGT allows for the selection and isolation of live cells grown onto thin polymer foil using a pulsing ultraviolet (UV) laser. The combination of LMDGT with a previously developed method for the visualisation of *Chlamydia* in live cells by staining with the Golgi-specific label BODIPY® FL C5-ceramide (Alzhanov et al., 2007; Hackstadt et al., 1995; Boleti et al., 2000), followed by the transfer of the label to bacteria, can be used to isolate single *Chlamydia*-infected cells. This combined technique has several advantages, including (i) that the search for cells of interest is performed under microscope control and (ii) that the isolation and collection of the selected cells is performed in a contact-free manner without causing the cells to detach from the base. The goal of this study was to test the utility of LMDGT for the isolation of single *Chlamydia*-infected cells for re-cultivation and for the whole-genome sequencing of *Chlamydia* derived from single inclusions.

2. Materials and methods

2.1. Host cell line and chlamydial infection

Chlamydia trachomatis serovar E (strain Bour) was cultivated in the HeLa cell line ATCC ® CCL-2 ™ (ATCC, Manassas, Virginia, USA). HeLa cells were grown in DMEM containing 4.5 g/L glucose and 10% foetal bovine serum (Life Technologies, Carlsbad, California, USA). Infection of the HeLa cell monolayer was accomplished via the inoculation of pure EBs. The elementary bodies were centrifuged onto cells at 1700 ×*g* for 1 h and incubated for another 2 h at 37 °C. After incubation, the infected cell monolayer was washed three times with Hanks' balanced salt solution (HBSS), after which culture medium was added and the monolayer was incubated in the ambient air with 5% CO_2 at 37 °C for 48 h. The new generation of elementary bodies was purified using urografin gradient ultracentrifugation, and a *Chlamydia* stock solution was prepared (Scidmore, 2005). The titer of the infectious progeny was determined by the infection of a sterile monolayer of HeLa cells with sequential dilutions of a *Chlamydia* stock solution, followed by the visualisation of the inclusions using direct immunofluorescence with the ready-to-use fluorescein-conjugated mouse monoclonal antibodies anti-*C. trachomatis* LOS (Nearmedic, Moscow, Russia). The titer of *Chlamydia* was measured in inclusion-forming units (IFUs).

2.2. Preparing infected cells for laser microdissection and labelling inclusions with BODIPY® FL C5-ceramide complexed to BSA

For the microdissection, 2 ml of a HeLa cell suspension (5000 cells/ml) were plated on a 50 mm membrane Petri dish (Leica Microsystems, Wetzlar, Germany) equipped with polyethylene naphthalate (PEN) foil (2 μm thickness) at the bottom. To improve the cell adhesion, the inside surface of the PEN foil was pre-coated with a 0.01% poly-L-lysine solution (Sigma-Aldrich, St Saint Louis, Missouri, USA). The membrane Petri dish was placed into a conventional plastic Petri dish with a diameter of 90 mm to avoid contamination of the outside foil surface. 24 h after cell plating, a chlamydial stock containing pure EBs was added to cells at a multiplicity of infection (MOI) of 0.1, centrifuged onto cells at 1700 ×*g* for 1 h, and incubated for another 2 h at 37 °C. The cells were washed with HBSS, and the culture medium was added. Infected cells were incubated in an ambient air with 5% CO_2 at 37 °C for 36 h. To visualise the infected cells (cells containing inclusions), the sample was washed three times in HBSS then incubated with BODIPY® FL C5-ceramide complexed to BSA diluted in HBSS (1:200; the stock solution was reconstituted according to the manufacturer's recommendations, Life Technologies) for 20 min at 37 °C. After staining, the cells were washed three times with HBSS and culture medium was added. The cells were then incubated for 30 min and provided with fresh culture medium without washing. The cells were then incubated for an additional 12 h. This step allows for the transfer of the fluorescent dye from the Golgi apparatus to the *Chlamydia* within the infected cells, while allowing for the efflux of the dye from the uninfected cells through exocytosis (Alzhanov et al., 2007; Hackstadt et al., 1995; Boleti et al., 2000). Before microdissection, the *Chlamydia*-infected cells were washed two times in 2 ml of HBSS. Then, 1 ml of fresh HBSS was added, and the membrane Petri dish without a cover was placed on the stage of the laser microdissection instrument.

2.3. Isolation of Chlamydia-infected cells by laser microdissection

In this study, we used the Leica LMD7000 laser microdissection system (Leica Microsystems), which was equipped with a climate chamber to provide temperature control. Individual live *Chlamydia*-infected cells were isolated by laser microdissection according to a previously described protocol for the isolation of single HeLa cells (Podgorny, 2013). To find the optimal parameters for the isolation and re-cultivation of single inclusion-derived *Chlamydia*, we performed experiments with different inclusion maturation times and different incubation conditions after the LMDGT procedure. The incubation conditions that were varied included the density of the HeLa cell monolayer within the strip-plate wells, the culture composition and the incubation time. The most successful conditions are presented here. Briefly, infected cells were visualised using the epifluorescence mode of the laser microdissection instrument by green fluorescence of BODIPY® FL C5-ceramide transferred to a chlamydial inclusion using the ×20/0.4 objective. Using the automatic 'Draw and Cut' mode of the microdissection software, we circumscribed a circular region with a 200 μm diameter around a single infected cell on the live image from the CCD camera, such that the selected region contained no other cells. The selected region was cut from the rest of the specimen using a pulsed UV laser. The laser settings for the cutting included a Power of 40 relative units (RU), an aperture of 1 RU, a speed of 6 RU, a head current of 100%, a pulse frequency of 120 Hz, and an offset of 120 RU.

After cutting, the sample did not immediately drop into the collector. Instead, we applied a laser pulse to the rim of the sample in the manual 'Move and Cut' mode of the microdissection software to release the sample into the collector. The laser settings for the release were a power of 40 RU, an aperture of 20 RU, a head current of 100%, a pulse frequency of 10 Hz, and an offset of 120 RU. The released sample then dropped into a well within an 8-well strip (Greiner Bio-One, Frickenhausen, Germany) with a pre-grown monolayer of HeLa cells. Isolated samples floating on the surface of the liquid were submerged by the addition of a droplet of culture medium to each well. Cycloheximide was added to the culture medium to a final concentration of 1 μg/ml. Each strip was inserted into a strip-plate, covered by a lid and placed into an incubator with 5% CO_2 and 37 °C to allow for the growth of new inclusions for a period of five to seven days. For genome sequencing, the sample was collected into the empty cap of a 0.5 ml Lobind tube (Eppendorf, Hamburg, Germany) and was immediately frozen at −20 °C until needed. To control for the homogeneity of the isolated samples, we analysed the sequences of the gene encoding MOMP.

2.4. Direct immunofluorescence for Chlamydia

Cell cultures infected with *Chlamydia* were fixed in 4% paraform in PBS pH 7.4 for 20 min at room temperature (RT) then permeabilised with 0.2% Triton X100 for 30 min at RT. The cells were stained with fluorescein-conjugated mouse monoclonal antibodies anti-*C. trachomatis* LOS for 1 h at RT. The host cells were visualised by

Download English Version:

https://daneshyari.com/en/article/2089897

Download Persian Version:

https://daneshyari.com/article/2089897

Daneshyari.com