Journal of Microbiological Methods 139 (2017) 37–44

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

Detection and quantification of intracellular bacterial colonies by automated, high-throughput microscopy

Christina L. Ernstsen[a,b], Frédéric H. Login[a], Helene H. Jensen[a,b], Rikke Nørregaard[a], Jakob Møller-Jensen[c], Lene N. Nejsum[a,*]

[a] Department of Clinical Medicine, Aarhus University, 8000 Aarhus, Denmark
[b] Department of Molecular Biology and Genetics, Aarhus University, 8000 Aarhus, Denmark
[c] Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense M, Denmark

ARTICLE INFO

Keywords:
Uropathogenic *E. coli*
UPEC
Quantitative high through-put imaging
Intracellular bacterial colonies
Pyelonephritis
Cystitis
Colony forming units
Gentamicin protection assay

ABSTRACT

To target bacterial pathogens that invade and proliferate inside host cells, it is necessary to design intervention strategies directed against bacterial attachment, cellular invasion and intracellular proliferation. We present an automated microscopy-based, fast, high-throughput method for analyzing size and number of intracellular bacterial colonies in infected tissue culture cells.

Cells are seeded in 48-well plates and infected with a GFP-expressing bacterial pathogen. Following gentamicin treatment to remove extracellular pathogens, cells are fixed and cell nuclei stained. This is followed by automated microscopy and subsequent semi-automated spot detection to determine the number of intracellular bacterial colonies, their size distribution, and the average number per host cell. Multiple 48-well plates can be processed sequentially and the procedure can be completed in one working day.

As a model we quantified intracellular bacterial colonies formed by uropathogenic *Escherichia coli* (UPEC) during infection of human kidney cells (HKC-8). Urinary tract infections caused by UPEC are among the most common bacterial infectious diseases in humans. UPEC can colonize tissues of the urinary tract and is responsible for acute, chronic, and recurrent infections. In the bladder, UPEC can form intracellular quiescent reservoirs, thought to be responsible for recurrent infections. In the kidney, UPEC can colonize renal epithelial cells and pass to the blood stream, either *via* epithelial cell disruption or transcellular passage, to cause sepsis. Intracellular colonies are known to be clonal, originating from single invading UPEC.

In our experimental setup, we found UPEC CFT073 intracellular bacterial colonies to be heterogeneous in size and present in nearly one third of the HKC-8 cells.

This high-throughput experimental format substantially reduces experimental time and enables fast screening of the intracellular bacterial load and cellular distribution of multiple bacterial isolates. This will be a powerful experimental tool facilitating the study of bacterial invasion, drug resistance, and the development of new therapeutics.

1. Introduction

Several pathogens including *Salmonella enterica*, *Listeria monocytogenes*, *Mycobacterium tuberculosis* and uropathogenic *Escherichia coli* (UPEC) are known to invade and colonize host cells, escaping host cell defense mechanisms. These infections can be challenging to treat, since their intracellular life-style often protects pathogens from immune system effectors. Successful infections include pathogen attachment to the host cells, subsequent invasion, and survival inside the host cell.

Cell culture models are frequently used to study the invasiveness and virulence potential of bacterial pathogens and to design intervention strategies. Following pathogen infection and invasion, the total number of invasive bacteria is counted by a gentamicin protection assay (Edwards and Massey, 2011; Negretti and Konkel, 2017), which is a modified version of the traditional colony forming unit (CFU) assay. Briefly, host cells are seeded in a cell culture dish and infected. Subsequently, the cell-impermeable antibiotic gentamicin is added to the medium post infection to eliminate extracellular bacteria while internalized bacteria remain unaffected. The samples are then homogenized and plated in dilution series for determination of intracellular bacterial titers. Although this method can be partially automated (Brugger et al., 2012), it remains time consuming and cannot be

* Corresponding author at: Department of Clinical Medicine, Aarhus University, DK-8000 Aarhus C, Denmark.
E-mail address: nejsum@clin.au.dk (L.N. Nejsum).

http://dx.doi.org/10.1016/j.mimet.2017.05.001
Received 17 February 2017; Received in revised form 1 May 2017; Accepted 3 May 2017
Available online 03 May 2017

paused, since it is live specimens that are measured. Furthermore, the number of samples that can be handled simultaneously is limited, and CFU determination provides only a crude measure of viable bacteria within the sample with no information regarding the size or the number of intracellular bacterial colonies per cell. Therefore, it is desirable to develop methods, where large number of samples can be handled simultaneously and where more information regarding intracellular bacterial colonization can be retrieved. Murphy et al. used an immunofluorescence staining method, followed by semi-automated counting of the total number of single intracellular and extracellular bacteria (Murphy et al., 2016).

Here we present an automated microscopy-based high-throughput method for quantifying number and size of intracellular bacterial colonies. Intracellular bacterial colonies formed by invading UPEC are clonal, formed from a single invading UPEC (Schwartz et al., 2011). The method presented allows measurements of the total number of intracellular bacterial colonies, the average number of colonies per host cell as well as the size of intracellular bacterial colonies. The total number of intracellular colonies reflects the number of UPEC which have invaded and the size reflects subsequent proliferation inside host cells.

Host cells are grown in 48-well plates and infected with fluorescent pathogens, followed by gentamicin treatment, fixation, and staining of host cell nuclei. The experiment can be paused after fixation. Imaging and image analysis by spot detection is semi-automated, and allows determination of the number of intracellular bacterial colonies, their size distribution, and the percentage of infected host cells. The use of automated spot detection and quantification considerably reduces the experiment time. Several 48-well plates can be handled, and the procedure is completed in a single working day. Thus, this approach constitutes a powerful tool for screening invasion efficiency and intracellular proliferation of multiple bacterial isolates as well as for studying their response to antimicrobial treatment (Blango and Mulvey, 2010).

To establish this analysis method, we used the prototypical UPEC strain CFT073 and immortalized human kidney cells (HKC-8) as a model system. UPEC can cause urinary tract infections (UTIs) and is known to colonize host cells by forming intracellular bacterial colonies.

UTIs are among the most common bacterial infectious diseases in humans. They are estimated to affect an average of 150 million people worldwide every year and pose a major economic burden on the health care system (Foxman, 2010; Stamm and Norrby, 2001). Upper UTIs are infections of the kidney (acute pyelonephritis) and ureters while lower UTIs correspond to infection of the bladder (cystitis) and urethra (Andersson and Michel, 2011). Cystitis is the most common form of UTIs. It is estimated that 40–50% of women will experience one episode of cystitis in their lifetime. Moreover, 25–30% of women who experience a primary infection will encounter a recurrent infection within six months (Foxman, 2003; Stamm and Norrby, 2001). Acute pyelonephritis can lead to renal damage and onset of chronic kidney disease.

Most UTIs in otherwise healthy individuals are caused by UPEC, which colonize tissues of the urinary tract, both extracellularly and intracellularly, and are responsible for acute, chronic and recurrent infections. UPEC enters the urinary tract through the urethra and migrates to the bladder using their flagellum (Wright et al., 2005). The subsequent bladder colonization relies on the ability of UPEC to adhere to the uroepithelium. Several pili appendages that protrude from the bacterial surface participate in UPEC adhesion and further invasion, where type 1 pili seem to be essential for infection (Connell et al., 1996). Type 1 pili are capped with the adhesin FimH, which interacts with surface-exposed mannosylated uroplakin (Zhou et al., 2001) and α3β1-integrins (Eto et al., 2007) on the bladder umbrella cells. These interactions lead to activation of a number of Rho-GTPases including Cdc42 and RhoA that in turn stimulate local actin rearrangement which promotes UPEC internalization (Martinez and Hultgren, 2002). Within the cytoplasm, UPEC rapidly divides to form intracellular bacterial communities (IBCs). These IBCs show biofilm-like properties and are protected from the immune system. Each IBC is clonally derived from a single UPEC, but several IBCs can form in one cell (Schwartz et al., 2011). Upon IBC maturation UPEC can exit infected bladder cells and invade neighboring cells to establish new IBC or ascend from the bladder to the upper urinary tract and cause acute pyelonephritis. Type P pili are critical for kidney invasion since the adhesin PapG located at the tip of these pili specifically interacts with glycosphingolipids of kidney cells (Karr et al., 1990; Stapleton et al., 1998). This interaction not only mediates adhesion of UPEC to the renal epithelium but also decreases the expression of the polymeric Ig receptor (PlgR) that transports IgA into the urinary tract, thus impairing the immune response (Rice et al., 2005).

It was proposed that the recurrent UTIs originate from quiescent intracellular UPEC reservoirs (QIRs) that form within the bladder mucosa. Unlike the rapidly expanding IBCs, QIRs consist of single bacterial cells or small communities of non-replicating bacteria that remain viable for months within bladder cells. The limited replicative activity of QIRs makes them virtually resistant to antibiotic treatment. Subsequent exit from dormancy may enable cells in QIRs to initiate a recurrent infection (Blango et al., 2014; Mulvey et al., 2001; Mysorekar and Hultgren, 2006). This is supported by a recent publication reporting the presence of intracellular UPEC reservoirs in bladder biopsies from female cystitis patients after completion of their antibiotic treatment although UPEC could not be detected in the urine (Liu et al., 2016). Thus, preventing intracellular UPEC proliferation and therapeutically targeting the dormant intracellular bacteria represent major challenges for antimicrobial treatment in order to efficiently treat and prevent recurring UTIs. Methods like the one described in this paper allowing detection and quantification of intracellular bacterial colonies are important tools for understanding and potentially targeting invasion and intracellular growth of both UPEC and other invasive pathogens.

2. Materials & methods

2.1. Plasmids, bacterial growth conditions, and cell culture

The uropathogenic *Escherichia coli* (UPEC) strain CFT073 (Mobley et al., 1990a, 1990b) isolated from a patient suffering from acute pyelonephritis was used for this study. CFT073 was grown at 37 °C in Luria-Bertani broth (LB) or on Luria agar plates.

The human proximal tubular epithelial cell line HKC-8 (Racusen et al., 1997) was grown in Dulbecco's Modified Eagles medium (DMEM) with 1 g/L glucose (Gibco, Copenhagen, Denmark) supplemented with 10% fetal bovine serum (FBS, Gibco, Copenhagen, Denmark) and 0.5 U/mL penicillin (Sigma, Copenhagen, Denmark), 0.5 g/mL streptomycin (Gibco, Copenhagen, Denmark), and 1 mg/mL kanamycin (Gibco, Copenhagen, Denmark) at 37 °C and 5% CO_2. Cells were kept in culture for up to 2–3 weeks.

2.2. Infection of HKC-8 Cells

To visualize the bacteria by fluorescent microscopy after cell infection, CFT073 was transformed with the pTurboGFP-B plasmid (Amp^r) that encodes TurboGFP (Evrogen), a bright and fast-folding version of green florescent protein (GFP). CFT073-GFP were grown overnight followed by GFP induction with 5 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (OD_{600} = 0.2, 2 h at 37 °C with 50 μg/mL of ampicillin). HKC-8 cells in 48-well plates were thoroughly washed with PBS to remove antibiotics (penicillin, kanamycin and streptomycin) from the culture medium and subsequently placed in the infection medium (DMEM low supplemented with 10% FBS, 50 μg/mL ampicillin and 5 mM IPTG). Cells were infected with CFT073-GFP at a multiplicity of infection (MOI) of 50. Plates were centrifuged for 5 min at 150 × *g* and incubated at 37 °C with 5% CO_2 for 1 h. To eliminate bacteria that remained extracellular, infected cells were washed twice