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Rapid detection of *Pseudomonas aeruginosa* by phage-capture system coupled with micro-Raman spectroscopy

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ABSTRACT

The early and accurate identification of medically relevant microorganisms like *Pseudomonas aeruginosa* is of great importance for human health. Current pathogen identification is routinely performed using conventional microbiological methods which are unable to detect fastidious organism that are difficult to culture and occur at low concentrations without time consuming multiple cultivation steps. This study reports the development of a novel rapid and cultivation-free method for highly sensitive and rapid detection of *Pseudomonas aeruginosa* based on the coupling of phage-capture system with optical techniques, namely FTIR and visible micro-Raman spectroscopies. Commercial latex beads were functionalized with the entire structure of engineered phage clones and used as bacterial capture and concentrating system. The rapid concentration of bacteria enhanced the detection of the Raman scattering signal by increasing the location concentration that is being processed. This method can be used to detect a low level of *P. aeruginosa* (10^3 cells/ml) from clinical samples without the use of selective media or additional biochemical tests. The sample testing process, including data acquisition, required a time less than one hour. The proposed system represents a proof of concept study for development of sensitive phage-based biosensors for rapid and specific one-step detection of pathogenic bacteria.

1. Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen which can cause a wide range of diseases such as nosocomial infection, pneumonia, cystic fibrosis, fester otitis media, urinary-tract infections in catheterized patients, sepsis and others [1–3]. Therefore, the development of rapid and sensitive technique for early detection of *Pseudomonas aeruginosa* is very important for effective treatment and prevention of those diseases. Current pathogen identification is routinely performed using conventional microbiological culture methods which require multiple cultivation steps and long incubation periods. These methods are time consuming (usually take 1–5 days) and have some limitations such as the low sensivity for fastidious micro-organisms that are difficult to culture. Recent molecular diagnosis methods including Polymerase chain reaction, fluorescent in situ hybridization and matrix-assisted laser desorption ionization–time of flight spectrometry can reduce the assay time to hours but they still have some disadvantages such as sample preparation procedure, amplification of the target microorganism and the need for DNA isolation and specific probes [4–6]. Recently, latex nanoparticles, functionalized with antibodies or synthetic peptides, have been utilized for a wide variety of applications [7–10]. However, the use of these bioactive molecules has some disadvantages like their sensitivity to physico-chemical variations of environment and their extremely high production and purification costs. Phage display is a high-throughput biotechnique for selection of specific molecular probes with high affinity against different targets [11]. This technology involves the introduction of exogenous peptide sequences into a location in the genome of the phage capsid proteins. The encoded peptides are expressed or "displayed" on the phage surface as a fusion product with one of the phage coat proteins. Filamentous phage particles are extraordinarily robust and stable, being resistant to heat, organic solvents, hard pH conditions and even 6 M urea [12]. Furthermore, the

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purified bacteriophage can be stored at moderate temperature without losing its infectivity and/or binding capability and their propagation on host bacterial cells is cheaper compared to both antibodies and peptides production. Thus, filamentous bacteriophages can act as antibody surrogates, possessing distinct advantages while achieving equivalent specificity and sensitivity [13]. In recent years, optical spectroscopies were used to discriminate among different bacterial strains. Since FTIR spectroscopy measures the vibration properties of the chemical bonds when excited by the absorption of the IR radiation, when applied to whole microbial cells, the infrared spectrum reflects the qualitative cells biochemical composition [14–16]. On the other hand, a Raman spectrum obtained from cells or tissues provides a molecular fingerprint of a specimen, yielding detailed information about molecular bonds, conformations and intermolecular interactions. It has high chemical specificity and molecular information can be obtained without requiring staining or labelling [17–20]. This vibrational spectroscopic technique has been used extensively to identify bacterial samples by a careful investigation of the vibrating modes of the molecules in the bacteria [21–23]. The Raman spectra of each bacteria is highly specific because each microorganism has a unique spectral pattern, which is the result of the vibrational information obtained from macromolecules of the bacterial cells. These detailed spectral informations are also related to phenotypic changes, resulting from environmental variations as well as cell heterogeneity due to cell processes such as cell growth, aging and metabolic rhythms [24,25]. However, in spite of its advantages, nowadays the major disadvantage is represented by the Raman low cross section of bacteria, allowing the acquisition of not well defined spectra, mainly when the target bacterial concentration is extremely low.

In this study, we propose the use of commercial latex beads functionalized with the entire structure of phage clones, selected through Phage Display technology, as capture and concentrating system for a single step detection of *Pseudomonas aeruginosa*. The captured bacteria were then detected by FTIR and visible micro-Raman spectra analyses. The proposed system represents a proof of concept study for the development of a sensitive phage-based biosensors for rapid and specific one-step detection of pathogenic bacteria.

2. Materials and methods

Pseudomonas aeruginosa ATCC 27853 was obtained from American Type Culture Collection (ATCC, LGC Promochem, Milan, Italy) and maintained on Luria Bertani (LB) medium. *TG1 Escherichia coli* was used for propagation of phage clones. Stock organisms were maintained in LB broth containing 20%(v/v) glycerol at −80 °C. P9b phage was derived from a M13 pVIII-9aa phage peptide library (kind gift of Prof. F. Felici). This clone displays the foreign peptide QRKLAAKLT, which represents a specific and selective probe for *Pseudomonas aeruginosa* in comparison with other Gram-negative and Gram-positive bacterial strains [26]. Carboxyl-polystyrene latex beads (diameter 0.8 μm) were purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). 1 ml of beads (10% w/v in ultrapure water) were mixed with 2.680 ml of 2-(*N*-Morpholino) EthanelSulfonic acid buffer (MES, 0.05 M pH 5.54), 20 mg of 1-Ethyl-3[3-Dimethylaminopropyl] Carbodiimide (EDC) and 320 μl of phage resuspended in MES buffer (title of 1.3×10^{12} PFU/ml). The sample was incubated in a tilting agitator (70 rpm) for 2 h at room temperature (RT). After the reaction, phage-coupled beads were concentrated by centrifugation at 2800*g* at RT for 15 min, washed twice with 4 ml of Phosphate Buffer Saline (PBS, 0.1 M pH 7.1) and then resuspended in PBS. With this procedure 2 ml of beads (5% w/v) are obtained. In order to verify phage coating of the beads, we performed an ELISA assay. 250 μl of functionalized beads were incubated with 1.750 ml of PBS+ 4% Bovine Serum Albumin (BSA) in order to block any residual uncovered sites on their surface for 1 h at 37 °C on a rotator mixer operating at 3 rpm. Then, the beads were concentrated by centrifugation at 2800 x *g* at RT for 15 min, washed three times with 1 ml of Washing Buffer (PBS + 0.05% Tween 20) and resuspended in 250 μl of PBS. 250 μl of anti-M13 major coat protein PVIII-HRP monoclonal conjugate (1:2500 diluted in PBS + 0.1% BSA + 0.5% Tween 20) were added. After one hour of incubation at 37 °C and three washing steps, reactions were developed with 250 μl of 3,3′,5,5′-TetraMethylBenzidine (TMB), Horse Radish Peroxidase (HRP) substrate, for 20 min in the dark, stopped with 31 μl of HCl 2 N and read at 450 nm in Multiscan FC (Thermo Scientific Type: 357). The negative control was represented by beads without P9b phage. The efficiency of capture was first assessed by Standard Plate Count. Capture tests were performed by incubating 20 μl of phage-coated beads with 1 ml of *Pseudomonas aeruginosa* (10^3 cells/ml in PBS) at 37 °C in orbital shaking at 220 rpm for 30 min. After centrifugation at 2800*g* at RT for 15 min the supernatants were recovered, and not captured bacteria were enumerated by spread in duplicate plating on LB agar plates followed by incubation overnight at 37 °C. The re-suspended bead pellet was also streaked on plates to obtain a further independent estimate of the concentration of unreleased cells. Colony Forming units per millilitre (CFU/ml) counts were determined before and after beads incubation with bacteria and the capture efficiency percentage was calculated. In order to determine maximum capture efficiency, 10^3 *Pseudomonas aeruginosa* cells/ml were tested against scalar concentration of functionalized beads (5,7.5,10 and 12.5% w/v) and results were reported as percentage average of captured bacteria. Then, the bacteria captured by the phage-coated beads were assessed by Fourier Transform Infrared (FTIR) and micro-Raman spectroscopies. The supernatant was withdrawn and the bead pellet was re-suspended in 20 μl of PBS and placed on a CaF_2 slide. The sample was allowed to dry in air before characterization. Negative capture control was represented by 20 μl of phage-coated beads without bacteria. The qualitative biochemical composition of the bacteria captured by the phage-coated beads was determined by Infrared absorption measure, carried out using a FTIR Spectrum 100 PerkinElmer spectrophotometer equipped with a microscope, operating in transmission and reflection mode, provided with Cassegrain optics, allowing to have a numerical aperture of 0.6, a 6X magnification, a focus spot of 0.25 mm and a Mercury Cadmium telluride detector. The image mode was used to collect the transmission spectra from several point in a $1000 \times 1000\ \mu m^2$ area, with 64 scans for pixel, $2\ cm^{-1}$ of resolution, in the 550–$4000\ cm^{-1}$ range, where CaF_2 absorption is weak. CaF_2 substrates coated with a very thin layer of bacterial cells were also placed in direct contact with an infrared attenuated total reflection diamond crystal whose spot is 10 μm. Our experiments were done in triplicates and ten spectra were acquired at RT. Each spectrum was composed of an average of 5 separate scans. The same sample was also detected by Raman spectroscopy in the 700–$1800\ cm^{-1}$ range using an Horiba XploRa spectrometer equipped with an Olympus BX40 microscope, a Peltier cooled charge coupled device (CCD) sensor and a 532 nm laser as the excitation source. Laser radiation was focused onto the sample to a spot of $0.7\ \mu m^2$ through the 100X microscope objective lens. The spectral resolution for the Raman measurements is $2\ cm^{-1}$. An acquisition time of 100 s allowed a sufficient signal/noise (S/N) ratio. To ensure reproducibility, experiments were performed in triplicate and no sample damage was observed. Ten spectra were acquired for each set of measurements and standard deviation of individual Raman peaks, with respect to mean value, was calculated.