Systematic and Applied Microbiology 39 (2016) 132–140

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Fast label-free detection of *Legionella* spp. in biofilms by applying immunomagnetic beads and Raman spectroscopy

Dragana Kusić[a], Petra Rösch[a,c,*], Jürgen Popp[a,b,c]

[a] Institut für Physikalische Chemie and Abbe Center of Photonics, Friedrich-Schiller-Universität Jena, Helmholtzweg 4, D-07743 Jena, Germany
[b] Leibniz Institute of Photonic Technology (IPHT), Albert-Einstein-Straße 9, D-07745 Jena, Germany
[c] InfectoGnostics Forschungscampus Jena e.V., Zentrum für Angewandte Forschung, Philosophenweg 7, D-07743 Jena, Germany

ARTICLE INFO

Article history:
Received 19 October 2015
Received in revised form 18 January 2016
Accepted 21 January 2016

Keywords:
Raman spectroscopy
Immunomagnetic separation
Biofilms
Legionella spp.

ABSTRACT

Legionellae colonize biofilms, can form a biofilm by itself and multiply intracellularly within the protozoa commonly found in water distribution systems. Approximately half of the known species are pathogenic and have been connected to severe multisystem Legionnaires' disease. The detection methods for *Legionella* spp. in water samples are still based on cultivation, which is time consuming due to the slow growth of this bacterium. Here, we developed a cultivation-independent, label-free and fast detection method for legionellae in a biofilm matrix based on the Raman spectroscopic analysis of isolated single cells via immunomagnetic separation (IMS). A database comprising the Raman spectra of single bacterial cells captured and separated from the biofilms formed by each species was used to build the identification method based on a support vector machine (SVM) discriminative classifier. The complete method allows the detection of *Legionella* spp. in 100 min. Cross-reactivity of *Legionella* spp. specific immunomagnetic beads to the other studied genera was tested, where only small cell amounts of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* compared to the initial number of cells were isolated by the immunobeads. Nevertheless, the Raman spectra collected from isolated non-targeted bacteria were well-discriminated from the Raman spectra collected from isolated *Legionella* cells, whereby the Raman spectra of the independent dataset of *Legionella* strains were assigned with an accuracy of 98.6%. In addition, Raman spectroscopy was also used to differentiate between isolated *Legionella* species.

Introduction

Legionella is a bacterium that replicates intracellularly within a wide range of protozoa and persists in the biofilms of water storage and distribution systems. About half of the members of the genus *Legionella* have been documented to be pathogenic, including *L. pneumophila*, which is the clinically most relevant species as it has been found to invade alveolar macrophages of the human lung causing the severe pneumonia of Legionnaires' disease [6]. Colonization of existing biofilms in water distribution systems by *L. pneumophila* appears to be dependent on the presence of other microbial species. For example, due to its intracellular lifestyle within protozoa, it has been shown to persist in biofilms without replication in the absence of amoebae such as *Hartmannella vermiformis* [21]. Other studies have suggested the extracellular growth of *L. pneumophila* within biofilms in the absence of amoebae [26]. In these studies, *L. pneumophila* has been considered in the context of pre-grown biofilms. However, the ability of this bacterium to form a biofilm itself in rich complex medium has been investigated by Mampel and colleagues [18]. It was found that clinical as well as environmental isolates of *L. pneumophila* were capable of forming biofilms, where the formation of a biofilm was found to be independent of the serogroup of *L. pneumophila* and widespread among *L. pneumophila* strains. Biofilm formation by *Legionella* species in nutrient-poor environments such as tap water has been presented in a recent study [16]. Compared to planktonic cells, sessile cells of *L. pneumophila* in biofilms accumulate polysaccharides as an extracellular polymer matrix as well as a greater amount of lipids. This finding has confirmed a distinct biofilm metabolism developed by *L. pneumophila* under low nutrient conditions. The ability of this pathogen to form biofilms in which the cells are surrounded by a self-produced matrix of diverse extracellular compounds may increase its survival in harsh environments and protect from harmful compounds.

* Corresponding author at: Institut für Physikalische Chemie and Abbe Center of Photonics, Friedrich-Schiller-Universität Jena, Helmholtzweg 4, D-07743 Jena, Germany. Tel.: +49 3641 948381; fax: +49 3641 948302.
E-mail address: petra.roesch@uni-jena.de (P. Rösch).

http://dx.doi.org/10.1016/j.syapm.2016.01.002
0723-2020/

Molecular detection tools such as Raman microspectroscopy or secondary ion mass spectrometry have been recently combined with isotope-labelling techniques for the analysis of a bacterial community to allow the examination of the metabolic properties of microorganisms at a single cell level [33]. Furthermore, many different rapid detection methods based on the separation and subsequent identification of bacteria in environmental samples have been developed. For instance, immunomagnetic separation (IMS) based on the specific antigen-antibody reaction in combination with other detection techniques such as double fluorescent staining, flow cytometry [10] or real-time PCR amplification [35] have been used for the detection of *L. pneumophila* in water. These techniques in combination with IMS require the use of highly specific monoclonal antibodies to detect the unique pathogen with a chosen specificity. Otherwise, accompanying cross-reactions of antibodies to similar epitopes of microbial species commonly found in environmental specimens limit the application of these methods for environmental work. In a recent study by Díaz-Flores and colleagues, a method based on IMS has been compared with the quantitative polymerase chain reaction (qPCR) as well as with traditional culture methods for the detection of legionellae in complex water samples [5]. The reported results have shown that the IMS method was less influenced by matrix effects and is thus more suitable for the analysis of complex water samples compared to both culture and qPCR [5]. IMS in combination with cultivation and flow cytometry was also used to investigate the occurrence of legionellae in both water and biofilm samples collected from groundwater wells [25]. It has been shown that the IMS method could be used to partially reduce background microorganisms thus allowing a more efficient isolation of legionellae from environmental samples [25].

The potential of vibrational spectroscopy for bacterial characterization has been documented [15,27–29]. So far, Raman microspectroscopy has been used as a fast and label-free method to identify isolated or extracted microorganisms from samples such as milk [19], meat [20], powder samples [30], urine [11], ascitic fluid [13] or sputum samples [12] without any cultivation. Here, various isolation techniques such as filtration, buoyant density centrifugation, and enzymatic milk clearing have been used to isolate bacteria from complex matrices. Furthermore, due to the high specificity of antibodies in the recognition of their target cells, immunocapture has been applied to separate and subsequently identify bacteria via Raman spectroscopy [9,22,34]. Likewise, surface enhanced Raman scattering (SERS) combining specific capture of bacteria by antibodies was used for the detection of pathogens [14,32]. Furthermore, for the identification of both *Salmonella enterica* and *Staphylococcus aureus* in complex food matrices such as spinach and peanut butter, Wang and colleagues proposed a MNPs@SiO_2-based SERS platform with a reported detection limit of 10^3 CFU/mL [34]. Knauer and colleagues have used an amino-polyethylene glycol-coated surface treated with *Legionella*-specific and *Salmonella*-specific antibodies to immobilize both *L. pneumophila* and *S. typhimurium* and to subsequently identify them with SERS. Furthermore, Jing and colleagues have demonstrated the possibility of using SERS to differentiate among infectious and mildly infectious strains of *L. pneumophila* [7].

The majority of diverse bacteria contain an almost identical molecular composition and display very similar Raman spectra. However, small differences in the molecular composition of various microbial cells result in a unique Raman signature allowing bacteria to be differentiated by their Raman spectra by applying chemometric approaches. It has been shown that the Raman spectra of numerous investigated legionellae can be differentiated via a multi class support vector machine [17]. In addition, Raman spectroscopy has been applied to discriminate between sessile cells of diverse *Legionella* species grown as monospecies biofilms [16]. However, obtaining the Raman spectra of single bacterial cells in environmental samples that can have a complex heterogeneous composition can be extremely challenging. For this, the application of Raman spectroscopy to identify distinctive bacterial species in environmental samples makes sense only if it is used in combination with an efficient isolation technique compatible to Raman spectroscopy [23].

In this study, an evaluation of IMS for its compatibility with Raman microspectroscopy to isolate bacteria from a biofilm matrix and their subsequent identification on a single cell level by applying multivariate analysis on the Raman spectra of single bacterial cells is presented. Here, commercial polyclonal antibodies specific to *Legionella* species were used to remove the cells of particular species out of the biofilm formed by that particular microbial species before Raman microspectroscopic analysis. Raman spectra collected from isolated single cells were used to train the classification model which was later validated by an independent Raman dataset. In addition, the recovery efficiency of the IMS isolation of the biofilm form of *Legionella* species was evaluated. Finally, cross-reactivity of immunomagnetic beads to non-target genera grown in biofilms as well as the potential of Raman spectroscopy to distinguish between them and *Legionella* species was estimated.

Materials and methods

Bacterial strains and growth conditions

Three subspecies of *Legionella pneumophila* representing serogroups 1, 4 and 5, (*L. pneumophila* ssp. *pneumophila* DSM 7513, *L. pneumophila* ssp. *fraseri* DSM 7514 and *L. pneumophila* ssp. *pascullei* DSM 7515), *Legionella bozemanae* DSM 16523, *Legionella micdadei* DSM 16640 *Legionella feeleii* DSM 17645, *Legionella dumoffii* DSM 17625 and *Legionella anisa* DSM 17627 obtained from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Germany) as well as *Escherichia coli* DSM 10806, *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* PAO1 provided by the Institute of Medical Microbiology, Jena University Hospital were used. An overview of the *Legionella* spp. and other bacterial species used throughout this study is provided in Table 1. Differential buffered charcoal yeast extract (DBCYE) agar (Sigma–Aldrich, D3560-500G) containing ferric pyrophosphate, L-cysteine hydrochloride and α-ketoglutarate was used to maintain bacterial cultures. All species were grown at 37 °C in a humid atmosphere (≈85% humidity) with 5% CO_2 for 24 h (*E. coli*, *K. pneumoniae* and *P. aeruginosa*) or 3 days (*Legionella* spp.) prior the biofilm development in filter sterile tap water so that the physiological state of the replicates was each time the same. For long-term storage, bacterial cultures were kept at −80 °C.

Formation of the biofilm samples

Bacterial colonies pre-grown on DBCYE agar were rinsed off with sterile distilled water, washed via centrifugation for 3 min at 5000 rpm at room temperature, and the bacterial pellet was then resuspended in sterile-filtered tap water (Pall Life Sciences, Acrodisc PF syringe filters with 0.2 μm Supor membrane) so that the final OD was approximately 1.5. Four mL of bacterial solution from each species was divided into 8 vessels (Lab-Tek™ chambered cover glasses, 734-2060) and incubated at 37 °C for 3 days in a humid atmosphere (≈85% humidity) with 5% CO_2 under static conditions to allow the biofilms to be formed at the borosilicate glass surface. The supernatant was afterwards carefully aspirated, while the remaining biofilms were carefully rinsed three times with distilled water. Washed biofilms were mixed thoroughly with sterile-filtered tap water by pipetting and frequently scratching the bottom of the vessel via an inoculation loop. Subsequently, the