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Research paper

Microbial identification from faces and urine in one step by two-photon excitation assay technique

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ABSTRACT

Two-photon excitation fluorometry (TPX) is a separation-free bioaffinity assay technique which enables accurate diagnostic testing in microvolumes. The technology is currently commercially applied in an automated mariPOC® test system for rapid phenotypic multi-microbe detection of pathogen antigens. The first TPX applications for diagnostics were intended for respiratory infection testing from nasopharyngeal and oropharyngeal samples. Feces and urine are more complex sample matrices and contain substances that may interfere with immunoassay binding or fluorescence detection. Our objective was to study the suitability of these complex matrices in the TPX technique. As expected, feces and urine elevated fluorescence levels but the methodology has the unique property of compensating for matrix effects. Compensation allows reliable separation of specific fluorescence from the fluorescence caused by the matrix. The studied clinical samples did not contain immunoassay inhibitors. The results suggest that the methodology is robust and may provide reliable testing of feces and urine samples with high accuracy.

1. Introduction

Respiratory tract infections and infectious diarrheal diseases are common causes of morbidity, especially in developing countries (Lozano et al., 2012). Both of the illnesses occur with varying severities. Major costs for the society are caused by work absences (Sacri et al., 2014). The etiological agent among otherwise healthy population often goes unconfirmed by laboratory methods (Tam et al., 2012). In vitro diagnostics is needed to identify the pathogen to specify proper medical care and for infection control (Peltola et al., 2005; Corcoran et al., 2014). Even differentiation between viral and bacterial etiology with high accuracy is usually impossible without specific in vitro diagnostics. The diagnostic testing needs to provide rapid results in order to be fully beneficial for the patient wellbeing by facilitating prompt initiation of proper medical care. Rapid testing also often lowers the overall health care costs (Bonner et al., 2003; Barbut et al., 2014) and helps to optimize antibiotic use (Llor et al., 2017). Also prevention of epidemics and accurate cohorting of patients, that are admitted to a hospital, are dependent on fast identification of the etiological agent (Kirby and Iturriza-Gómara, 2012; Borrows and Turner, 2014; Barclay et al., 2014). Therefore, there is a huge clinical need for easy to use, affordable and highly accurate multianalyte tests that can be used in decentralized settings.

Application of two-photon excitation fluorometry in bioaffinity assays (Hänninen et al., 2000) has enabled the development of an automated diagnostic test system for rapid multianalyte antigen detection that can be used at the point-of-care or in on-call laboratories. This platform is sold under mariPOC® brand name (ArcDia International Ltd., Turku, Finland). This next generation antigen detection test system combines accuracy of laboratory antigen testing and rapidity (Koskinen et al., 2007). The test system currently provides differentiation of up to twelve pathogens (http://www.maripoc.com) causing respiratory infection or tonsillitis symptoms and five pathogens for intestinal infections. The average specificity of respiratory infection tests has been reported to be higher than 99% in clinical studies. Most of the positive cases are reported in 15–20 min, while laboratory level accuracy results are reported latest in 1–2 h. The test system is regarded as easy to use with hands-on time less than a minute for nasopharyngeal swab samples (Ivaska et al., 2013; Tuuminen et al., 2013; Sanbonmatsu-Gámez et al., 2015).

The technology applied by mariPOC® is known as separation-free ArcDia™ TPX (two-photon excitation) assay technique. It is a fluorescence-based bioaffinity assay technology which allows detection of biomolecules from microliter volumes without compromises in assay accuracy and repeatability. The signal yield in the detection is independent of the reaction volume (Hänninen et al., 2000). The

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technique has shown wide applicability in the detection of biomolecules in different sample matrices, such as detection of mucosal antigens (Koskinen et al., 2007), serum antigens (Hänninen et al., 2000; Koskinen et al., 2004) and antibodies (Koskinen et al., 2006), antibody avidity (Smolander et al., 2010), and nucleic acid sequences (Meltola et al., 2005; Vaarno et al., 2004). The technique has also shown its efficiency in phenotypic antimicrobial susceptibility testing directly from polymicrobial patient samples (Koskinen et al., 2008; Stenholm et al., 2013). The latter application combines in well culture, in the presence and absence of antimicrobials, and real-time immunometric detection of pathogen-specific growth. The application is fast version of suspension culture and broth microdilution susceptibility testing. The difference is that the new approach does not necessitate bacteria in pure cultured (isolated) format. Ability to provide identification and antibiotic susceptibility in one-step is possible due to the specific antibodies and the separation-free immunoassay detection by the TPX technique. The assay yields signals proportional to bacterial growth which, in turn, is dependent on antibiotic resistance profile of the targeted bacteria.

In the mariPOC® applications for respiratory infections the sample is taken from the nasopharynx or the oropharynx. However, sampling from the respiratory tract is not the only way to detect pathogens causing respiratory infections. *Legionella pneumophila* and *Streptococcus pneumoniae* are pneumonia causing bacteria which are often tested by detecting their pathogen specific carbohydrate antigens from urine, usually by immunochromatographic or enzyme-linked immunosorbent-based assays.

Suitability of the urine or stool sample matrices for TPX assay technique has not been reported in the scientific literature before. Urine and feces are the waste products of our digestive tract excreted by two separate routes. Both of the matrices are complex and containing similar substances, such as inorganic salts, urobilinoids (decomposition products of hemoglobin) and albumin (Athar et al., 1999), that might interfere with the TPX fluorescence measurement by attenuating or enhancing fluorescence. The matrices also contain substances that can potentially interfere, e.g. inhibit or cause unspecific binding, with the bioaffinity reactions of immunoassays (Yolken and Stopa, 1979). Separation-free assay format brings benefits in the format of simple assay protocols, but it also means that the problematic sample matrix cannot be washed away before completing immunoassay reaction and fluorescence readout.

In this study, we explored the applicability of the TPX assay technique for the detection of antigens from urine and feces. Proof of concept tests were developed for *L. pneumophila* and adenovirus from urine and feces, respectively.

2. Materials and methods

2.1. Assay principle

The new assay method for antigen detection from feces or urine is based on immunometric assay principle and separation-free ArcDia™ TPX bioaffinity assay technique (Soini et al., 2002). The methodology uses polystyrene microparticles as a solid phase carrier coated with antibodies specific for the antigen, and fluorescent antibody conjugates as a tracer. When the reagents are incubated with a sample containing specific antigens, three component immunocomplexes are formed, resulting in locally concentrated fluorescent antibody conjugates on the surface of the microparticles. Immunocomplex formation is directly and quantitatively proportional to the concentration of the antigens in the sample.

Two-photon excited fluorescence is measured from the solution phase (F_S) and from the individual microparticles (F_{MP}) using an ArcDia™ TPX analyzer (Soini et al., 2002; Koskinen et al., 2004). The measurement is done through the transparent bottom of a 384 well plate (or other plate or cartridge format). The excitation wavelength is near-infrared (1064 nm), two simultaneous photons exciting the fluorophore, and emission takes place in visible wavelengths at around 560 nm. Due to the nature of two-photon process, the excitation of fluorescence takes place only in the focal volume, which is about the size of the microparticle used in the assay as a solid phase carrier (Hänninen et al., 2000). The reaction well is scanned for microparticles by deflecting the laser beam. A microparticle entering the focus results in increased backscattering at 1064 nm which is detected using confocal arrangement. This backscatter reveals the analyzer that a particle is being scanned for fluorescence brightness. The apparent brightness of the particle depends on the degree of bioaffinity binding and solution matrix (unbound tracer and sample matrix). The microparticle is pushed through the focus by optical forces during a time frame of about tens of milliseconds (Soini et al., 2002), after which the scanning continues. The signal from the solution phase (no backscatter detected) reflects unbound tracer and the sample matrix (Hänninen et al., 2000; Koskinen et al., 2007). The ratio of microparticle signal to solution signal approximates to unity in samples that do not contain target antigen.

2.2. Reagents

Fluorescent TPX succinimidyl ester labeling reagent was prepared as described previously (Meltola et al., 2004). Monodisperse, carboxyl-modified microparticles (diameter 3.22 µm; 11.3% [wt/vol]; 1.2 carboxyl acids/nm2) made of cross-linked polystyrene were purchased from Bangs Laboratories (Fishers, IN). Microtitration plates (384-well plate with black walls and a clear bottom, 788,096) were obtained from Greiner Bio-One (Frickehausen, Germany), and plate sealing film (adhesive PCR film, AB-0558), was obtained from Abgene Thermo Scientific (Massachusetts, United States of America). All biochemical reactions were performed in mariPOC® RTI sample buffer (B02, ArcDia International Ltd).

2.3. Preparation of immunoassay reagents

Monoclonal antibody (clone A1) against *Legionella pneumophila* serotype 1 (ArcDia International Ltd) was coated onto monodisperse, carboxyl-modified microparticles by using passive coating and EDAC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide] fixation. The tracer antibody was prepared by conjugating the antibody with a succinimidyl ester of the fluorescent labelling reagent by using methods described previously (Waris et al., 2002). Anti-adenovirus microparticles and tracer were prepared similarly with clones B1 and B2, respectively (ArcDia International Ltd). Assays in this study were conducted using soluble reagents. To demonstrate proof of concept for one-step assay protocol, the reagents were dried on 384-well plate wells as described previously (Koskinen et al., 2005). Activity of the dried reagents was compared to soluble reagents.

2.4. Samples

Analyte-free pooled urine was prepared by combining seven urine samples, including one morning urine and six daytime urines from six healthy donors. Four routine diagnostics surplus urine samples which had been positive in binaxNOW® *Legionella pneumophila* serotype 1 lateral flow test were obtained from Eastern Finland Laboratory Centre Joint Authority Enterprise, Mikkeli, Finland. Analyte-free Bristol scale type 3 stool sample was from a healthy donor. Four adenovirus antigen immunochromatographic test positive and ten negative Bristol type 6 stool samples were obtained from Vaccine Research Center, University of Tampere, Tampere, Finland.

2.5. Assay procedure

The immunoassay procedure is described previously by Koskinen et al. (2007). The assay reagent cocktails were prepared into the RTI