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.

Keywords: Two-photon excitation fluorometry, Separation-free immunoassay, Feces, Urine, Infectious diseases, Rapid testing

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 minutes, while laboratory level accuracy results are reported latest in 1-2 hours. 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 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^{TM} 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^{TM} 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, 788096) 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 sample buffer by mixing the anti-legionella or anti-adenovirus antibody coated microparticles and the corresponding tracer in final concentrations of 5,000 pieces/µL and 1.0 nmol/L, respectively.

Purified native carbohydrate extract of *L. pneumophila* serotype 1 antigen (ArcDia International Ltd), purified adenovirus type 2 hexon antigen (ArcDia International Ltd) and all sample dilutions were made into the RTI sample buffer. The analyte-free and Legionella antigen positive urines were diluted by a factor of two up to 128 times. The stool samples were vortexed vigorously, diluted in proportion of 1:5 in volume and centrifuged for 5 minutes at 1,000 g to remove solid and particular material. The analyte-free stool supernatant was diluted by a factor of two up to 512 times. Recoveries were studied by mixing legionella and adenovirus into corresponding sample matrices at concentrations of 140 ng/mL and 32 ng/mL, respectively. Adenovirus antigen-positive stool supernatant was diluted 0, 2, 3, 12, 48 and 192 times for the dilution series. All the other thirteen stool samples were analyzed using 20-fold dilution. Ten microliters of urine or stool dilutions were mixed with ten microliters of the analyte specific assay reagent mixture as duplicates into the reactions. After sealing the wells, the plate was inserted into a TPX analyzer

for incubation and fluorescence readout. Cross-reactions were studied as described in supplement (SUPPLEMENTARY 1).

3. Results

3.1. Analytical verification of the test methods

Reagents for the legionella method were developed in this study. Due to the nature of the study extensive optimizations were not performed, but reaction parameters known to provide sufficient performance were chosen based on past experience with previously developed tests. Reagents for adenovirus were developed previously.

Analytical sensitivities of the legionella and the adenovirus methods in buffer conditions were defined by the interception of the dose response curve and the cut offs (three times the standard deviations of negative controls). In 2 hours, the reactions had essentially (> 90% maximum signal) reached equilibrium. The immunoassay reactions reached half of the maximum signal within a half hour. The analytical sensitivity of the legionella method was 0.5 ng/mL for *L. pneumophila* serotype 1 antigen. For adenovirus method the analytical sensitivity was 0.7 ng/mL for adenovirus hexon protein trimer. The analytical sensitivity of the adenovirus method corresponds to approximately $1x10^5$ viruses in 20 µL sample volume, when one adenovirus consists from 240 hexon trimers (molecular weight 327 kDa, UniProtKB entry P03277).

Analytical specificities of the methods were studied by challenging them with high concentrations of other microbes. Neither method did cross-react with the studied viruses or bacteria. All signals were below respective cut offs.

The effect of drying of the test reagents on 384-well plate was studied by comparing the signal of dried reagents to those obtained with soluble reagents. The dried reagents provided 90-110% signal compared to the soluble reagents. Intra assay precision was lower than 10% with both reagents.

3.2. The effect of stool and urine matrix in the TPX fluorescence measurement

In order to study the effects of stool and urine matrices in the separation-free TPX fluorescence assay technique, a dilution series from both matrices was prepared into the RTI sample buffer. Diluted urine or stool supernatant was mixed together with the reagent mixture for legionella or adenovirus, respectively. Fluorescence from the reaction wells was measured kinetically without any separation of unbound and bound reagent fractions or potentially interfering matrix. The data was analyzed for the effect of sample matrices. According to the results, when the analyte was not present in the sample, stool supernatant and urine elevated both immunocomplex specific microparticle and solution fluorescence signals as a function of matrix concentration (Fig. 1). When the solution fluorescence background was subtracted from the microparticle fluorescence signal obtained from the same well, the subtraction (F_{MP} - F_S) was around zero and subtracted fluorescence remained clearly below cut off in all analyte-free urine and stool dilutions. For our experiments the cut off values were determined as three times the standard deviation of reactions with the highest matrix concentration (two times dilution) of the analyte-free urine or stool. Higher fluorescence levels caused by the matrix were observed from the stool samples (up to 9,000 cps) compared to the urine samples (up to 3,000 cps) and the buffer reactions (200 cps). Only a slightly higher microparticle fluorescence compared to solution fluorescence (ratio 1.13) was observed with the lowest stool dilution. The matrix fluorescence

levels were elevated similarly when the experiment was repeated without the fluorescent tracer antibody. This meant that the small unspecific signal originated from two-photon excited fluorescence of the compounds in the stool matrix and not from unspecific binding of the tracer due to the matrix compounds.

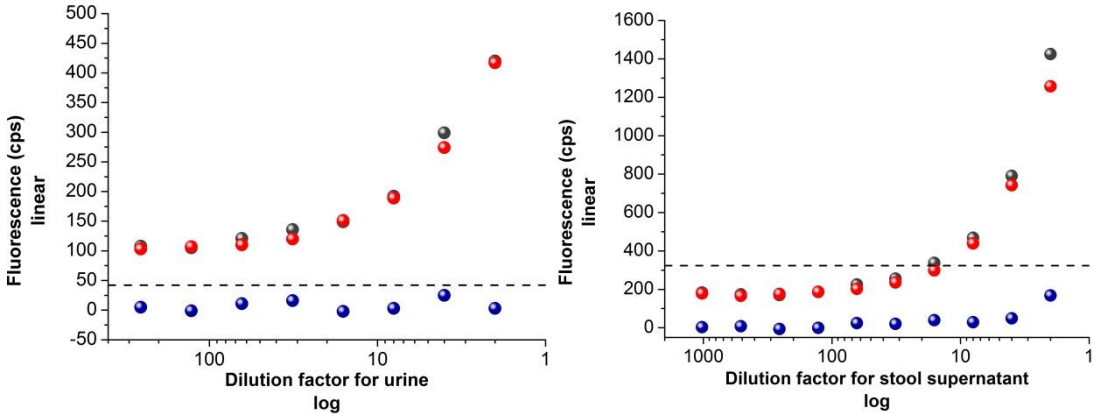

Fig 1. The effect of analyte-free urine (left) and stool (right) on TPX fluorescence levels in different dilutions. Fluorescence measured from the microparticles and the solution phases are shown as black and red dots, respectively. Specific fluorescence (F_{MP} - F_S) is shown as blue dots. Dashed lines indicate the defined cut offs (three times the standard deviation of the analyte-free urine or stool diluted by a factor of two).

3.3. Specific antigen detection in the presence of urine or stool matrix

Analytical recoveries from the urine and stool matrices were studied by spiking legionella antigen into analyte-free pooled urine dilutions and adenovirus antigen into stool sample dilutions. Antigen concentrations were kept equal in all dilutions. The recovery of legionella antigen (16 ng/mL) from urine was around 200% when urine was diluted 1:1 in RTI sample buffer. This unexpected recovery was observed with different antigen concentrations and in several repeated experiments. Unspecific binding was excluded by omitting the antigen from the reaction. The recovery declined to 100% when urine was diluted more than 32 folds. The recovery of adenovirus antigen 70 ng/mL) from stool was 85%, 88% and 99% when the stool matrix was diluted by a factor 10, 20 and 80, respectively.

Optimal sample dilution and ability to detect infection originated antigen from urine and stool was studied with reference test positive samples in a dilutions series. The legionella method detected all of the four antigen positive samples. The signal change with respect to sample dilution was linear (Fig 2.). The highest specific fluorescence signals were observed with the lowest urine dilutions. Therefore, there was no indication for immunoassay inhibition.

The dilution series for adenovirus positive stool sample showed a typical hook-effect of separation-free assays (Fig 2.). The hook effect results from a very high antigen concentration where microparticle antibodies are crowded with the antigen. The excessive antigen in solution blogs the tracer antibodies from attaching to solid phase captured antigens. Accordingly, formation of three component immunocomplexes is inhibited. The antigen concentration in the original stool sample was estimated to be 0.7 milligrams per gram of stool based on comparison to dose response of a standard antigen. The rest of the stool samples were analyzed using 20-fold dilution. The three other adenovirus antigen positive stools were also high positives and the ten adenovirus negative samples were tested negative.

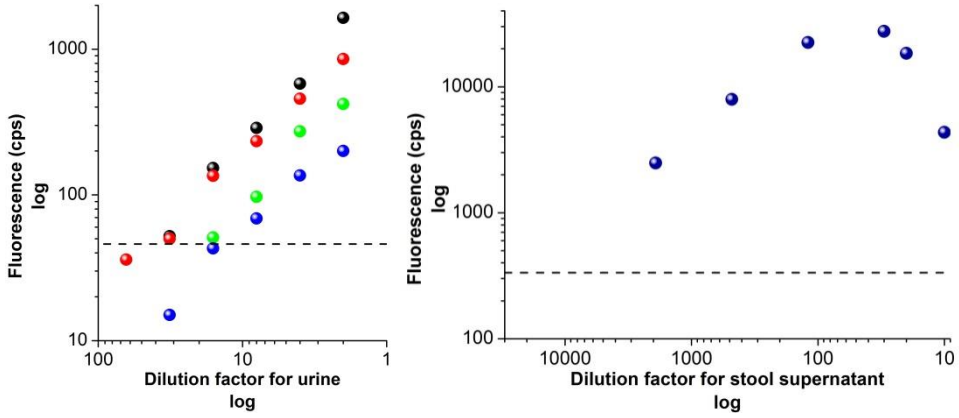

Fig 2. Dose-response plots for Legionella antigen positive urines in legionella method (left) and adenovirus antigen positive stool in adenovirus method (right). On the left, the four urine samples are presented with different colors. Fluorescence signals are presented as subtracted (F_{MP} - F_S). Dashed lines indicate the defined cut offs (three times the standard deviation of the analyte-free urine or stool diluted by a factor of two).

4. Discussion

4.1. Suitability of urine and feces for TPX assay technique

In acute infection testing, rapidity, ability for multianalyte detection, ease of use and high accuracy have been desired properties of point-of-care diagnostic tests for decades. However, despite such solutions have become recently available, new methods are still slowly deployed in rapid decentralized testing. This is probably due to cost issues and that new diagnostic methods are typically implemented first in laboratories. The laboratory use of rapid diagnostics compromises the potential benefits of tests with short turnaround time because sample logistics takes time. Also the diagnostic methods for routine testing of acute gastroenteritis are moving towards non-culture based methods, e.g. detection of antigens or nucleic acids (Jones & Gerner-Smidt 2012), and hence more suitable for decentralized testing.

In this article we introduced a proof of principle for using a novel automated rapid testing platform for infectious disease diagnostics from urine and stool samples. The platform brings decentralized testing a significant leap closer to the desired diagnostic properties. The TPX fluorescence signal is proportional to the concentration of the target molecule in the sample. Therefore, the new methodology enables quantitative results and novel research about the correlation between clinical manifestations and pathogen concentrations in urines and feces.

In this study a method for the detection of *L. pneumophila* serotype 1 antigen was developed. The legionella and adenovirus tests were verified to be analytically specific in urine and stool matrices, respectively. The tests did not cross-react with other tested microbes. The tests showed good precision and analytical sensitivity with purified standard antigens. The pathogen specific immunometric methods (antibody reagents) would likely work also in other platforms such as enzyme-linked immunosorbent assay or immunochromatography but the achievable sensitivity is expected to be lower than with the fluorescent TPX technique.

We showed that microbial antigens can be detected from urine and stool by the TPX assay technique without wash steps. Sample preparation for urine analysis was simple. Only dilution to the sample buffer was required. The stool samples needed the removal of solid material by centrifugation. Best performances were achieved when the sample dilution factors were two for

urine and ten to forty for stool. Higher dilution is needed for the stool to bring the analyte concentration into assay range and decrease the solution fluorescence.

4.2. Matrix effects

Significant inhibition of fluorescent excitation, emission detection or immunoreaction was not observed. As hypothesized, stool and urine contained substances that elevated fluorescence levels in the TPX measurements. However, the methodology has a unique property to compensate matrix effects. Matrix affected equally the solution and the microparticle fluorescence signals. Thus, the fluorescence caused by the matrix can be eliminated in its simplest format by reduction of the fluorescence signals. This can be done on any level of matrix fluorescence. Thanks to the compensation, matrix fluorescence caused by urine did not affect significantly the test sensitivity. Three times higher matrix fluorescence, on average, was observed for stools compared to urines. Stool seems to require higher dilution and clinical cut off value than urine to ensure high specificity. Higher cut off value will reduce sensitivity (compared to assays performed in buffer conditions) but only slightly. The mechanism by which urine caused higher than 100% apparent recovery is so far unknown and should be studied further.

Substances that fluoresce in the TPX measurement were not studied in detail but we hypothesize that most of the matrix fluorescence, other than tracer related, is caused by bilirubin-albumin complexes that are known to be fluorescent in the TPX assay system (Meltola et. al., unpublished data). Bilirubin and its albumin complexes have broad fluorescence emission in the range of 500-600 nm (maximum at 528 nm) when excited at 487 nm (Athar et al., 1999). Similar emission profile is also expected under two-photon excitation. The emission of a bilirubin-albumin complex slightly overlaps with the emission of the fluorescent tracer used in TPX measurements. Normal concentration of bilirubin in urine is 5 µmol/L (Putnam, 1971) which is about four logs higher than the fluorophore concentration used in our prototype tests (0.5 nmol/L). Despite relatively small sample dilutions, the fluorescence levels obtained in the presence of the urine and stool matrices elevated only slightly compared to the signal obtained from the tracer only. This is probably because the excitation of bilirubin-albumin complex is weak and/or the optical band filter filtrates the fluorescence efficiently. Fluorescence could be also caused by other urobilinoids than bilirubin or some other substances as inorganic salts with fluorescence properties. The magnitude of matrix fluorescence might be reduced by optimizing the used detector, filters and used fluorescent label molecule, e.g. among those described by Meltola et al. (2004). In addition, a sample pretreatment that eliminates fluorescent compounds or the fluorescence of the compounds in urine and feces would diminish fluorescent background.

4.3. Limitations

The limitations of our study include the limited number of clinical samples analyzed. Thus, we were not able to fully study sample to sample variation in signals caused by urine and stool matrices. For this reason, we may have missed interfering factors that are not systematically present in all samples, such as heterophilic antibodies or immunoassay inhibitors. The diagnostic accuracy of the developed methods could not be defined because of limited number of specific analyte positive samples. We were not able to test cross-reactions against other *Legionella* species or serotypes than *L. pneumophila* serotype 1. However, cross-reaction with other *Legionella* species or serotypes would be beneficial in clinical diagnostic use. This study did not enable to study in detail which substances caused elevated fluorescence levels in two-photon excitation and if their effect could be further eliminated or minimized. Nevertheless, we were able to show that the TPX technique platform is a worthy alternative when developing new stool or urine diagnostics.

5. Conclusions

In conclusion, urine and stool sample matrices seem to suit well in the TPX assay technique. The compensation of matrix effects by the TPX assay technique seems to allow antigen testing from stool or urine with high accuracy and specificity. mariPOC® platform could bring automation, multianalyte tests, as well as laboratory level accuracy and quality control to rapid diagnostics of urine and stool samples. The multianalyte applications could cover detection for viruses, bacteria, biomarkers and, potentially phenotypic bacterial antimicrobial susceptibility testing.

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Conflicts of interest
The authors are employees in ArcDia International Ltd.

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Author contributions

JMK: Major contribution in scientific designing and executing the studies, result analysis, scientific analysis and writing the manuscript.
JMS: Substantial contribution in scientific analysis of the fluorescent phenomena and revising the results and the manuscript critically.
NM: Substantial contribution in preparing the fluorescent molecules, scientific analysis of the fluorescent phenomena and revising the results and the manuscript critically.
JOK: Substantial contribution in scientific designing and result analysis, analysis of the fluorescent phenomena, study designs and writing the manuscript.

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SUPPLEMENTARY 1

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

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Studied cross-reactions

In this study, analytical specificities of the legionella and adenovirus methods were studied by excluding cross-reactions against other microbes. Viruses were purified viral culture preparations expect for rotavirus and norovirus which were partly purified suspensions from stool samples. Viruses were used in high concentrations at least 10^9 viruses/mL. Viral concentrations were based on results obtained from specific tests. Bacteria were cultured on plate and the colonies were harvested into saline. Optical density at 600 nm was measured. When optical density was 1.0 the bacterial concentration was considered as 10^9 bact/mL. Reaction concentrations for the bacteria were 10^6 and 10^8 bact/mL.

Cross-reactivity of the legionella method was studied against the following microbes:

Streptococcus agalactiae
Streptococcus bovis
Streptococcus pneumoniae, serotypes 1, 3, 4, 6B, 7F, 9V, 14, 15A, 18C, 19A, 19F, 23F
Streptococcus mitis
Streptococcus mutans
Streptococcus pyogenes
Streptococcus sanquis
Streptococcus anginosus
Streptococcus constellatus
Staphylococcus aureus
Staphylococcus epidermis
Staphylococcus intermedius
Haemophilus influenza
Haemophilus parainfluenzae

Cross-reactivity of the adenovirus method was studied against the following microbes:

Adenovirus type F40
Rotavirus A (serotypes G1, G2, G3, G4 and G9)
Norovirus GII.4
Norovirus GI.4
Astrovirus
Human bocavirus 1
Influenza A virus
Influeza B virus
Human metapenumovirus
Parainfluenza 1 (Sendai)
Parainfluenza 2
Parainfluenza 3
Respiratory syncytial virus

Staphylococcus aureus
Staphylococcus epidermidis
Streptococcus agalactiae
Streptococcus bovis
Streptococcus pneumoniae, serotype 4
Streptococcus pyogenes
Streptococcus mitis
Streptococcus mutans
Streptococcus sanguinis
Haemophilus influenza
Haemophilus parainfluenzae
Aeromonas hydrophila
Bacillus cereus
Campylobacter hyoilei
Campylobacter lari
Campylobacter fetus ss. Fetus
Campylobacter jejuni
Campylobacter coli
Clostridium perfringens
Clostridium difficile
Escherichia coli
Helicobacter pylori
Salmonella typhi
Salmonella enteritidis
Salmonella typhimurium