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A multipronged approach for systematic *in vitro* quantification of catheter-associated biofilms

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ABSTRACT

Biofilms are a leading cause of infections, especially those initiated on medical tubing. Quantification of pathogenic biofilms is critical for systematic determination of infections, antibiotics prescription, and implant replacement. By the time infectious pathogens are detected in fluids such as blood or urine, substantial and potentially life-threatening biofilms are likely to have already formed. These biofilms can have broad ranging patient care and cost implications. Here we propose a non-strain-specific protocol combining four microbiological assays, which are rarely used together in clinical biofilm assessment, to accurately quantify cellular and extracellular components attached to medical surfaces. Our results demonstrate that the shortcomings of standard approaches can be overcome through the conjunct analysis of total protein (modified Lowry), total biomass (quantitative polymerase chain reaction), cellular activity (ATP luminescence), and extracellular polymeric substances (Periodic Acid-Schiff assay). The efficacy of this multipronged approach was verified using four pathogenic, clinical isolates (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, methicillin resistant-*Staphylococcus aureus*, and *Candida albicans*) and two types of silicone catheters *in vitro*. Despite the variation of biofilm matrices among infectious agents, this approach comprehensively quantified the pathogenic load as well as fouling resistance in indwelling catheters obtained from patients. The sensitivity, reproducibility, multi-species specificity, and high-throughput potential makes this approach valuable for quality assessment of catheters, implants, and ventilators in hospital settings.

Introduction

Chronic infections are associated with microbial growth in the form of adhered colonies surrounded by large exopolysaccharide matrices, which can lead to hazardous biofilms (Doyle, 2000). Due to phenotypic changes or mutations, biofilms are less susceptible to host defenses such as macrophage phagocytosis and can become resistant or tolerant to antibiotics, resulting in reduced treatability (Bjarnsholt, 2013). Medical tubing is susceptible to biofilm formation and is often the cause of severe infections, especially because it serves as a hiding place for microbes where the immune system is less effective (Trevisani et al., 2005; Stickler, 2014). For example, urinary catheter tubing is associated with over 75 % of urinary tract infections, which are the most common health care-associated infections (HCAIs) (CDC, 2015).

The current 'gold standard' used to evaluate the presence of infections on medical tubing is culturing from urine. However, because catheter-associated urinary tract infections are often preceded by biofilm formation, it would be advantageous to make an earlier, faster, and more direct measurement of adhered microorganisms to assist in the diagnosis and prevention of clinical infections (Percival et al., 2015). Additionally, the diagnosis potential that can be achieved by using planktonic cells that have sloughed off from biofilms is limited, which has important healthcare implications for accurate diagnosis and appropriate treatment. For instance, studies have shown that when isolating *Staphylococcus* strains from medical stents, forty-six strains were isolated from the stent segment cultures while only seven were isolated from urine samples for the same set of patients (Gad et al., 2009).

Current standard methods target: 1) Viable cells via plate counting or flow cytometry, 2) Total biomass via optical density, 3) Extracellular

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polymeric substances (EPS) via resazurin dye, crystal violet dye, or live/dead staining, or 4) Cellular activity via ATP quantification (Stepanović et al., 2000; Stepanović et al., 2007; Doll et al., 2016; Singh et al., 2017). Reliable detection by these assays is limited by factors such as small-colony variants, non-culturable microorganisms, and false positives resulting from the inability to distinguish live from dead cells. Additionally, many current methods evaluate planktonic cells rather than biofilms even though the data are not directly transferrable (López Pérez et al., 2017), while they also lack in QA/QC methodologies. These limitations, along with the fact that the methods are rarely performed together, prevent accurate quantification or identification of problematic biofilms. Single pronged approaches, such as those listed above, are not generalizable for all strains since the composition of biofilm is not universal among all microorganisms (Stepanović et al., 2000; Stepanović et al., 2007; Doll et al., 2016; Singh et al., 2017; López Pérez et al., 2017).

Due to the widespread occurrence of biofilms and their negative effects on patient outcomes (Gomila et al., 2019), catheters were used as model surfaces to test our approach of quantifying biofilms. The goals of this study were to: 1) optimize the extraction protocol of biofilms from catheter segments; 2) formulate a protocol for biofilm quantification that provides data on both the cellular constituents and the extracellular matrix; and 3) develop a multipronged approach that is reproducible, sensitive, culture independent, and is suitable for clinical settings as a result of valid QA/QC methodologies.

Materials and methods

Chemicals

Dextran (dextran from *Leuconostoc* spp. Mr ~40,000. 31389, Fluka) was obtained from MilliporeSigma (Burlington, MA). Acetic acid (99.7 %, ACS grade) and Luria Bertani (LB) broth (BD Difco) were obtained from Sigma-Aldrich (St. Louis, MO).

Pure strain clinical isolates and catheter conditions

In vitro experiments were carried out using common microbial pathogens encountered in catheter-associated urinary tract infection patients (Mittal et al., 2009; Gad et al., 2009; Behzadi et al., 2015). Clinical isolates of *Staphylococcus aureus*, methicillin resistant--*Staphylococcus aureus* (*MRSA*), *Pseudomonas aeruginosa*, and *Candida albicans* were previously isolated from urine preserved in boric acid by UCLA Department of Pathology and Laboratory Medicine. Ten catheter samples, which had resided in male patients for 2–30 days, were obtained in collaboration with Cedars-Sinai Urology Department. Once removed, the catheters were placed on ice and transported to UCLA for further analysis (additional detail in SI).

Design of in vitro experiments and detachment of adhered biofilms

Catheter preparation and incubation

In vitro experiments were performed by cutting the catheter tubing into 1-inch segments (16 Fr diameter, 5.3 mm, Type: Foley) and placing each segment into a sterile, 20 mL glass scintillation vial (Fig. S1). Silicone catheters and silicone catheters modified with a hydrophilic coating (additional detail in SI) were selected due to their widespread clinical use (Feneley et al., 2015) and reduced biofilm formation susceptibility (Lin et al., 2019; Yan et al., 2020). After each 1-inch coated or uncoated catheter segment was placed into a scintillation vial, it was submerged in 10 mL of tryptic soy broth with an OD_{600} of 0.1 and allowed to incubate at 37 °C with 120 rpm shaking. After the initial 24 h, 9 mL of culture were removed and replenished with 9 mL of LB broth to ensure sufficient nutrient content. The samples were then allowed to incubate for an additional 24 h. After the total 48 -h incubation period, the catheters were removed and dried by rolling the full segment's perimeter on sterile absorbent paper and gently tapping each edge of the tube to remove any excess liquid, loosely bound cells, and liquid adhered to the inner lumens. Each catheter segment was then transferred into 5 mL of sterile deionized (DI) water in 15 mL Falcon tubes.

Biofilm extraction

The extraction of tightly bound cells and EPS components was carried out as previously described by Mandakhalikar et al. (2018). Validation of successful extraction of EPS and QA/QC are provided in the publication by Mandakhalikar et al. (2018) including visualization with electron microscopy and mixed model statistics. Falcon tubes containing sterile DI water and the dried catheter segments were vortexed continuously at full speed (10 speed) for 1 min. The samples were then subjected to 50 s of probe sonication (QSonica, Newtown, CT) at 20 kHz and 12 V amplitude. Finally, samples were vortexed for another minute (10 speed). After the vortex-sonication-vortex sequence was completed, the catheter pieces were removed from the tube and the remaining suspension was used to carry out subsequent analyses. Optimization parameters of this method included the use of 16 Fr diameters instead of 3–14 Fr diameters, 1 inch vs 1 cm segments, and probe sonication duration.

Analytical methods

Total nucleic acids extraction and quantitative polymerase chain reaction

Extraction of nucleic acids was carried out using a phenol-chloroform extraction method as previously described (Gedalanga et al., 2014). For cell density measurements, 500 µL liquid samples were collected after incubation, with cells harvested via centrifugation (21, 000 x *g*, 10min at 4C) and the supernatant was discarded. Further details are presented in the SI. The number of cells was determined by amplification of the 16S rRNA or 18S rRNA taxonomic genes. Primer sequences are listed in Table S1. Quantitative Polymerase Chain Reaction (qPCR) with SYBR-green-based detection reagents were utilized to quantify gene copy numbers in each isolate. Each plate consisted of a calibration curve with a template-free control. Melt curve analyses were performed after each run to ensure single product amplification.

Modified Lowry assay for protein quantification

Tightly bound protein measurements were accomplished with the Pierce Modified Lowry Protein assay (Fisher Scientific, Pittsburgh, PA), using bovine serum albumin as the standard (1 mg/L – 1500 mg/L). 40L of sample or standard were combined with 200 µL of Modified Lowry Reagent and incubated for 10 min. Subsequently, 20 µL of 1X Folin-Ciocalteau Reagent was amended and the plate was re-incubated for 30 min. Absorbance was measured at 750 nm using VICTOR 3 V plate reader (PerkinElmer, Waltham, MA) at 1 s intervals in a 96-well clear bottom, opaque-walled plate.

Adenosine triphosphate (ATP) assay for cell viability

The adenosine triphosphate (ATP) concentration was measured using the BacTiter-Glo™ Microbial Cell Viability assay (Promega, Madison, WI). A calibration curve was prepared from lyophilized luciferase (Sigma-Aldrich) ranging from 0.0057 mg/L to 5.7 mg/L. 100 µL of cell sample and 100 µL of BacTiter-Glo reagent were pipetted into a 96-well opaque flat bottom and opaque-walled plate. Samples were incubated at room temperature for 5 min and analyzed for luminescence using the spectrophotometer plate reader mentioned above. Background luminescence was determined by following the same procedure as experimental samples but with sterile deionized water and BacTiter-Glo reagent. In order to account for variations in clinical isolate samples, bound ATP was normalized to the amount of ATP in the corresponding supernatant as shown below in Equation 1.

$$\frac{BoundATP_{cathetersegment} - BackgroundATP_{deionizedwater}}{SupernatantATP_{cathetersegement} - BackgroundATP_{deionizedwater}}$$

Periodic acid-Schiff assay for carbohydrate quantification

The polysaccharide fraction, as calibrated by dextran, was extracted from samples using a modified carbohydrate extraction method as described previously (Kilcoyne et al., 2011; Randrianjatovo-Gbalou et al., 2016). For this assay, 25 µL sample or standard were pipetted into a 96-well plate with 120 µL of freshly prepared solution containing 0.06 % periodic acid in 7% acetic acid with gentle pipette mixing. Then a cover was secured to the multiwell plate, paraffined and incubated for 30 min at 37 °C with 60 rpm shaking. After incubation, 100 µL of room temperature Schiff reagent was pipetted into each well, mixed via tapping the well plate, and incubated again at 37 °C for 20 h to allow for color development. Absorbance was read in a 96-well clear bottom, opaque-walled plate at 550 nm using the spectrophotometer plate reader mentioned above. Dextran, a homopolymer of glucose, had a standard solution range from 210 mg/L to 50,000 mg/L.

Statistical analyses

Statistical differences between the coated and uncoated catheters were determined by a two-tailed, two-sample *t*-test that assumes equal variance. All experiments were performed in triplicate with analytical duplicates or triplicates. The results are presented as the mean ± the standard deviations (SD). The values were deemed statistically significant at p-values < 0.05. QA/QC parameters for the proposed assays and methodology which may be used to ensure accuracy by other laboratories or hospitals are presented in Tables S2 and S3.

Results and discussion

The enumeration of DNA in extracellular polymeric matrices attached to catheters is a critical parameter that facilitates quantification of microbial growth, adhesion, and overall biofilm integrity. Extracellular DNA (eDNA) typically originates from cell lysis and/or active or passive extrusion mechanisms (Nagler et al., 2018; Pietramellara et al., 2009). eDNA has been shown to be a universal adhesive substance and aids in biofilm formation (Okshevsky and Meyer, 2015; Okshevsky et al., 2015), which is why this parameter is of great importance when trying to quantify biofilm presence and growth. qPCR is a rapid and targeted quantification method that can accurately detect various nucleic acid biomarkers (Kralik and Ricchi, 2017).

qPCR amplification of universal gene targets effectively identifies and enumerates nucleic acids, yet this technique remains largely absent from methods striving to quantify adhered biofilms on medical surfaces. Additionally, virulence gene targets, which play a role in biofilm formation by affecting quorum sensing, surface adhesion, and other mechanisms, have been identified for pathogenic organisms, such as the *icaADBC* gene cluster (Gad et al., 2009) in *Staphylococcus* species and the *las* cluster in *Pseudomonas aeruginosa* (Malešević et al., 2019) (S1.3). However, the specificity of these targets may make them less successful at accurately indicating patient microbial load compared to universal gene targets (Fig. S3), since these are able to target highly conserved and reliable sites within the genome via the 16S or 18S primers.

Cycle threshold (C_T), which is proportional to the -log of the nucleic acid concentration, is reported because of its use in clinical settings to diagnose patient infections (Wellinghausen et al., 2009; Héry-Arnaud et al., 2017; Stenehjem et al., 2012). Results demonstrated that within the adhered biofilm, bacterial or fungal DNA was successfully detected across all undiluted catheter segment samples, but that the ability to accurately quantify the DNA decreased when samples were diluted 10-fold and 100-fold to represent clinically-ambiguous copy numbers (Figs. 1A, 2A, Fig. S2, Table S2). *MRSA* and *P. aeruginosa* clinical isolates showed the greatest overall surface attachment to uncoated catheters both with an average C_T value of 15.1 (Fig. S2). EPS contains nucleic acids in the form of RNA and eDNA (Yin et al., 2019). Studies have shown that several species, including *S. aureus* and *P. aeruginosa*, can utilize eDNA to promote or modulate the development of biofilms (Okshevsky and Meyer, 2015; Allesen-Holm et al., 2006). Microbial cells have also been shown to prefer hydrophobic surfaces when developing a biofilm (Doyle, 2000). Our results showed that bound DNA was significantly greater on the uncoated silicone segments compared to coated hydrophilic segments across all clinical isolates tested. This could be attributed to the fact that eDNA enhances adhesion to hydrophobic surfaces due to the amphiphilic nature of DNA (Das et al., 2011b). Correspondingly, *P. aeruginosa* showed the most significant difference between coated and uncoated catheters (p-value < 0.01).

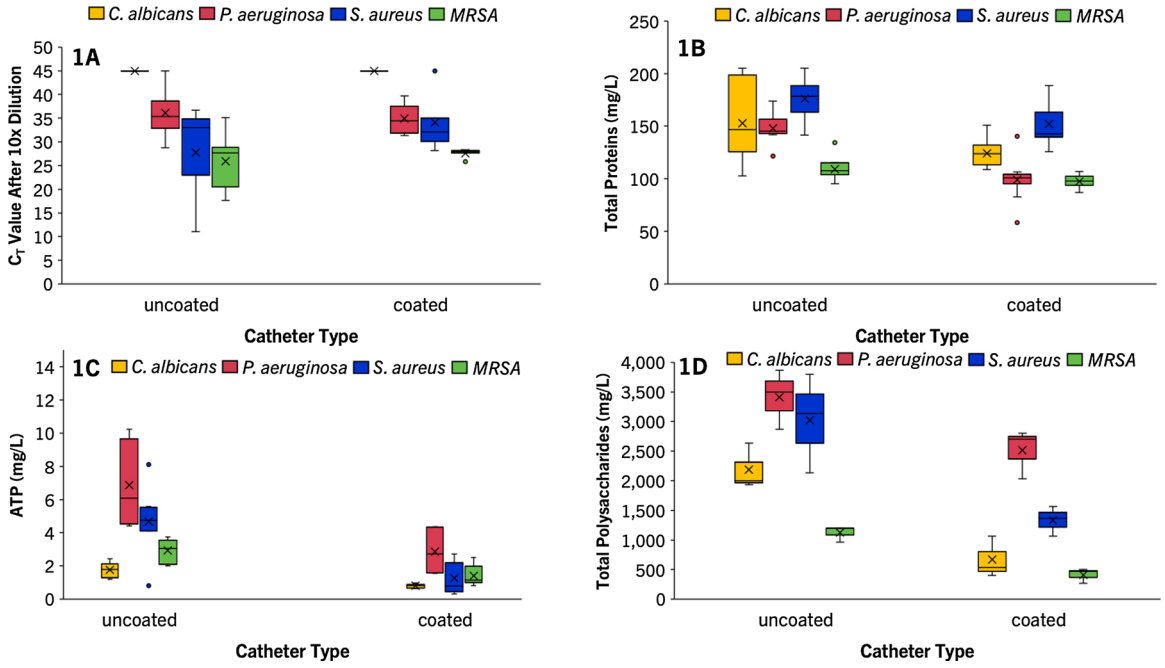

Fig. 1. Gene abundance, total protein, ATP, and total polysaccharide concentrations from adhered biofilms on coated and uncoated catheter segments. A). Gene abundance represented by C_T values. B). Total protein concentration as estimated via a bovine serum albumin standard. C). ATP concentration. D). Carbohydrate concentration as µg equivalents of dextran. Each value represents average and quartiles of experimental triplicates, each measured thrice (n = 9).

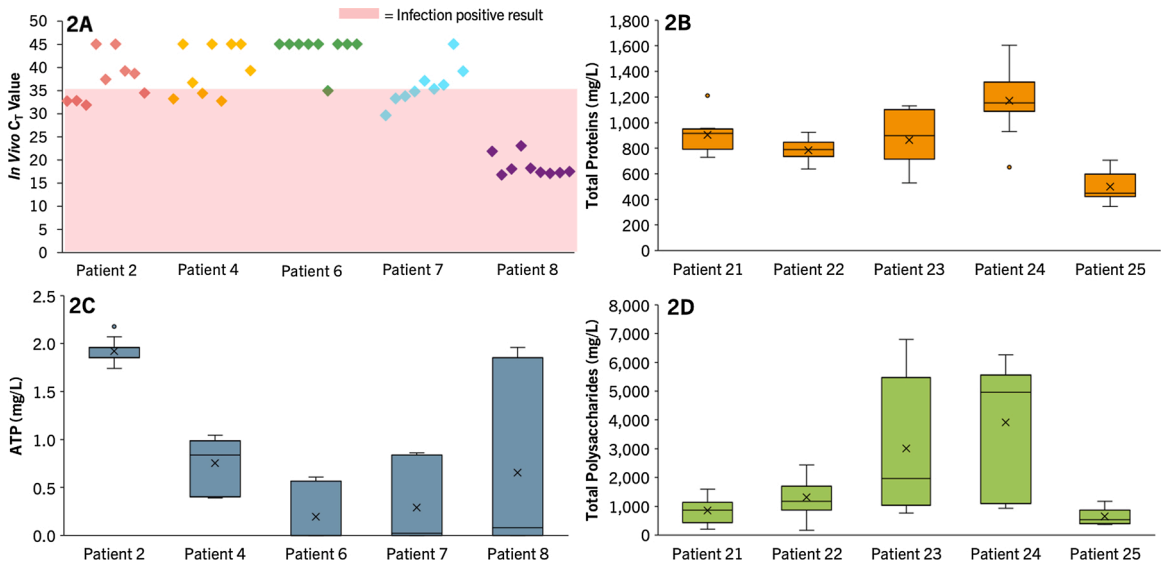

Fig. 2. Quantification of gene abundance, total polysaccharides, ATP, and total proteins adhered to uncoated catheters. A) Bacterial or fungal gene abundance represented as C_T values. The shaded pink area indicates infection positivity threshold, meaning, a C_T value within the shaded pink area could indicate a positive infection result for a patient (Wellinghausen et al., 2009; Héry-Arnaud et al., 2017; Stenehjem et al., 2012). B). Total Lowry protein concentration. C). ATP concentration. D). Carbohydrate concentration as μg equivalents of dextran. Each patient represents average and quartiles of experimental triplicates, each measured thrice (n = 9).

Quantifying proteins in the biofilm of pathogenic microorganisms is an important metric because it provides indicators of components contributing to adhesion, as well as to an infection's virulence (Cucarella et al., 2004; Gad et al., 2009; Fong and Yildiz, 2015; Wong et al., 2012; Stauder et al., 2012; Bhowmick et al., 2008; Cramton et al., 1999; Patel et al., 2012). Protein components of biofilm include secreted extracellular proteins, protein subunits of pili or flagella, proteins of outer membrane vesicles, and cell surface adhesins (Fong and Yildiz, 2015).

The modified Lowry method was selected because it has been shown to be the optimal protein quantification method for natural biofilms when compared to others such as the Bicinchoninic acid (BCA) or Bradford Coomassie protein assays. For example, the Lowry assay has a lower detection limit than both the BCA and Coomassie assays. Additionally, the Coomassie assay has been shown to underestimate the concentration of glycoproteins, and the BCA assay shows interference with saccharides and lipids (10 mM–100 mM) (Richards et al., 2020; Fountoulakis et al., 1992). Contrary to these methods, the modified Lowry method's major interfering factors are not major components of biofilms but are instead anthropogenic compounds such as detergents, chaotropes, and reducing agents, which are unlikely to appear in biofilms (Noble and Bailey, 2009). Furthermore, all reagents used in the Lowry assay are stable at room temperature, granting it a relatively low susceptibility to experimental error or reagent degradation.

Total protein content measured by the modified Lowry assay showed greater protein association to uncoated catheter segments compared to coated segments (Figs. 1B & 2B). The greatest amount of protein on unmodified catheters corresponded to *S. aureus* with values of 176 ± 19.6 mg/L, while the least amount of protein was measured for *MRSA* (98 ± 6.8 mg /L) on coated catheters. Differences between amounts of protein measured among strains may indicate differences in their ability to colonize surfaces, but also result from the fact that the composition of extracellular matrix varies between species, as well as among strains of the same species (e.g., *MRSA* vs. *S. aureus*) (Floyd et al., 2017). The greatest difference between uncoated/coated catheters occurred with *P. aeruginosa* (p-value < 0.01). *P. aeruginosa* proteomics have shown that the EPS of this species contains mostly outer membrane proteins, but also includes cytoplasmic, secreted, and periplasmic proteins, which are directly linked to adhesion and biofilm stability (Fong and Yildiz, 2015).

ATP plays an essential role in cellular respiration, metabolism, and energy storage (Mempin et al., 2013). The ATP concentration within a biofilm matrix can provide insight into the performance of the biofilm and overall cellular energy (Heffernan et al., 2009). Viable microbial load within the biofilm was quantified via the BacTiter-Glo cell viability assay (Sánchez et al., 2013). Previous studies have shown that there is a linear relationship between intracellular ATP and the number of viable cells (Kapoor and Yadav, 2010; Herten et al., 2017), which is why this assay was selected to characterize and quantify biofilm production. This luminescence-based assay enables the detection of ATP in catheter-associated biofilms.

ATP concentrations were successfully quantified across all samples and showed that greater ATP was associated with the uncoated catheters than the coated catheters (Figs. 1C & 2C). Coated catheter segments had significantly less ATP than uncoated segments across all clinical isolates. *P. aeruginosa* showed the greatest difference between the coated (6.88 ± 2.44 mg/L) and uncoated (2.87 ± 1.22 mg/L) catheters. This could be linked to the fact that the phosphate kinase gene (PPK), which encodes for the inorganic phosphate in ATP in *P. aeruginosa*, is responsible for thick and differentiated biofilm formation (Rashid and Kornberg, 2000). Contrastingly, *C. albicans* had the lowest overall concentrations of ATP on the catheters, but still showed significantly more ATP on uncoated catheter segments (1.77 ± 0.45 mg/L) than coated catheter segments (0.81 ± 0.13 mg/L). These results are consistent with Haghighi et al. (2012) in which the ATP assay was used to measure the ability of TiO_2-coated catheters to resist *C. albicans* adhesion.

Quantifying total carbohydrates is essential in evaluating the presence of biofilm because EPS is the most abundant component of the extracellular matrix of many biofilm-forming pathogens (Floyd et al., 2017). Additionally, polysaccharides are integral constituents in determining biofilm structure and integrity (Colvin et al., 2013). For instance, one of the prevalent carbohydrates in *P. aeruginosa* facilitates cell-to-cell adhesion within the biofilm by crosslinking eDNA within the extracellular matrix (Floyd et al., 2017). Carbohydrate quantification is thus considered a crucial component in the evaluation of biofilms due to their significance for biofilm stability.

Detection and analysis of complex carbohydrates is typically performed via colorimetric assays, including phenol-sulfuric acid (Masuko et al., 2005), Monsigny resorcinol (Monsigny et al., 1988), resazurin (Peeters et al., 2008) and crystal violet (O'Toole, 2011). The Periodic

Acid-Schiff reagent (PAS) is widely used to visualize tissues by staining glycogen and other polysaccharides (Kilcoyne et al., 2011; Speranza et al., 1997; Bock et al., 2007). In contrast to other methods, the PAS assay does not face much interference from proteins or sugars, has the ability to capture neutral and charged carbohydrates, and is not specific to any kind of glycosidic linkage (Kilcoyne et al., 2011; Randrianjatovo-Gbalou et al., 2016). This is important when concerned with quantifying carbohydrates in real biofilms because there are many carbohydrates present in EPS, even within the EPS of a single species (Floyd et al., 2017). For instance, studies on the extracellular matrix of *P. aeruginosa* have shown that different strains secrete as much as three types of matrix exopolysaccharides (Floyd et al., 2017; Hatch and Schiller, 1998; Jackson et al., 2004; Yang et al., 2007; Zhao et al., 2013). Ensuring the universality of the assay is critical for its widespread clinical applicability. Kilcoyne et al., (Kilcoyne et al., 2011) developed a method to use the PAS assay in a microtiter plate format for the *in vitro* quantification of dissolved carbohydrates (Kilcoyne et al., 2011), introducing the possibility to use it as a high-throughput method. However, this method is yet to be explored as a strategy for quantifying biofilms extracted from indwelling medical tubing.

Results from the PAS assay showed that the concentration of bound carbohydrates was significantly greater in uncoated catheter segments compared to coated ones (Figs. 1D & 2D. *P. aeruginosa* had the greatest amount of bound carbohydrate on coated catheters (3411 ± 505 mg equivalent dextran/L), while *MRSA* had the lowest concentration (411 ± 126 mg equivalent dextran/L) on coated catheters. *S. aureus* showed the greatest difference between uncoated/coated segments (p-value $<$ 0.001). The linearity of the response of a number of polysaccharides to the assay indicates (Kilcoyne et al., 2011) that it can be reliably used for different strains regardless of the dominant carbohydrate in their EPS. Thus, in contrast to other methods, the selected assay is not only useful for a large range of microorganisms but can also be confidently used with microbial communities, such as those present in clinical settings. Additionally, the PAS assay requires low sample volume and generates small amounts of non-hazardous waste, so it has the potential to be used as a high-throughput assay for biofilm quantification in clinical settings.

Fig. 2 shows that all four assays were successful at detecting and quantifying polymicrobial biofilms extracted from catheters that were previously implanted in patients. Gene abundance has been used to diagnose *S. aureus*, *C. albicans*, and *P. aeruginosa* infections via C_T value (Wellinghausen et al., 2009; Héry-Arnaud et al., 2017; Stenehjem et al., 2012) and ATP has been used to estimate antimicrobial effects (Sánchez et al., 2013; Schwarz et al., 2005). Based upon existing infection guidelines, patients 21 and 22 could be diagnosed to not have an infection relatively easily with only qPCR, since the samples were distant from the infection positive threshold and consistent for all replicates (Fig. 2A, Table S3). Correspondingly, the average ATP and total polysaccharide concentrations in patient 21 and 22 were substantially lower than the other patients. Contrastingly, for patients 23 and 24, the distribution of points for 16S rRNA gene abundance could be considered inconclusive in the diagnosis of an infection since the replicate analyses straddle the threshold (Fig. 2A). In these instances, additional data would be beneficial. For example, patient 23 had the highest average ATP (11.1 μg/L), while patient 24 displayed the highest average total polysaccharides (3911 mg/L) and total protein (1171 mg/L) (Fig. 2B–D). The ATP, polysaccharides, and protein concentrations are convergent with the qPCR results and add another dimension to the enumeration of biofilm material on catheters. The ability of the four methods to provide accurate quantification of proteins, nucleic acids, carbohydrates and ATP regardless of the specific chemical composition of the biofilm makes the approach valuable in the quantification of real clinical biofilms which are made up of a variety of microorganisms since the non-specificity of the reactions involved in each assay prevents detrimental biases.

In summary, this study emphasizes a multiple lines of evidence methodology for directly characterizing and quantifying catheter-associated biofilms *in vitro*, an approach which is superior to the current methods relying on culturing of planktonic cells in biological fluids (Tables S4 and S5). When implemented in a clinical setting, these assays may collectively aid the accurate enumeration of infections and inform costs and patient care decisions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hazl.2021.100032.

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