

Comparison of quantification methods for an endoscope lumen biofilm model

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ABSTRACT

Biofilm has been implicated in multi-drug resistant organism outbreaks following endoscopic procedures. Automated Endoscope Reprocessors (AER) are devices validated to clean and disinfect endoscopes per applicable standards. The ISO 15883 part 4 standard guides performance testing validation of AERs, including cleaning performance using a biofilm test soil. The standard recommends assessment of biofilm reduction using protein or carbohydrate quantification methods. The aim of this study was to assess the suitability of various quantification methods using the ISO biofilm model.

The ISO 15883 part 5 biofilm test soil method was used to grow biofilm within lumens representative of endoscopes channels. The biofilm was then quantified using five methods: Crystal Violet (CV), Colony Forming Units (CFU), Total Organic Carbon (TOC), protein assay with Orthophthalaldehyde (OPA), and protein assay by micro bicinchoninic acid (μ BCA). The five methods were statistically analyzed for their ability to assess biofilm reduction on samples accurately and precisely. In addition, the quantification methods were compared to demonstrate statistical equivalency, and thus their suitability for assessing biofilm cleaning performance testing of AERs.

1. Introduction

Biofilms are a major vector of healthcare-associated infections (HAI) [1–3]. They pose a particular challenge due to their persistence on surfaces, especially on reusable medical devices. Reusable thermolabile medical devices, such as endoscopes, are at high risk for harboring biofilm. They are manufactured with materials that prevent the devices from being sterilized using thermal methods such as steam. Additionally, the small channel diameters, delicate internal and external instrumentation, and complex designs provide locations that can harbor bacteria and favor the formation of biofilms [4]. Reprocessing of flexible endoscopes can include an automated step using Automated Endoscope Reprocessors (AERs) prior to storage or sterilization. AERs are intended to perform automated cleaning and high-level disinfection of endoscopes. The performance validation of AERs is highly regulated and must follow strict standards and guidelines (i.e. ISO 15883–4 [5], ISO 15883–5 [6], ANSI/AAMI ST91 [7], FDA [8]). International standard ISO 15883–4:2018 clause 6.11.3.2 a) requires that the cleaning efficacy

of AERs shall be validated on surrogate devices (representative models of endoscope channel designs) contaminated with a biofilm test soil [5, 6]. The biofilm test soil described in ISO 15883–5 is used to validate performances of an AER process to clean biofilm in endoscope lumen. The test intends to demonstrate that the parameters reached in the endoscope's lumen during the cleaning phases of the AER cycle allow for effective removal of biofilm. Biofilm removal testing on real devices cannot be performed due to the destructive nature of the test. Consequently, the standard allows for the use of endoscope lumen surrogate devices that reproduce a worst-case of endoscopes internal design. The method to produce biofilm on endoscope surrogate devices was introduced by Pineau et al. in the late 1990s and is well characterized for clinical relevance [9]. It consists of growing *Pseudomonas aeruginosa* under laminar flow in Polytetrafluoroethylene (PTFE) tubing for 72–96 h. The samples processed in the AER are then compared to a positive, unprocessed control to determine biofilm reduction. The ISO standard suggests using colony forming units (CFU), protein, or carbohydrate quantification to estimate the amount of biofilm on the samples. However, biofilm quantification techniques have evolved in the past decade.

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Abbreviations

AER	Automated Endoscope Reprocessor
CFU	Colony Forming Unit
CV	Crystal Violet
FDA	United States Food and Drug Administration
HAI	Healthcare-Associated Infections
ISO	International Standards Organization
LR	Log reduction
OPA	Orthophtalaldehyde
PR	Percentage reduction
PTFE	Polytetrafluoroethylene
SD	Standard Deviation
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TOC	Total Organic Carbon
μBCA	Micro bicinchoninic acid
UP	Ultra Pure

Azeredo et al. reviewed 12 methods of biofilm quantification, assessing their applications, advantages, and limitations [10]. Yet, several of these methods are either costly, not sensitive, time consuming or not accessible for industrial applications.

In this study, we compared five methods for biofilm quantification: protein using Pierce micro bicinchoninic acid (μBCA) assay, protein using an orthophtalaldehyde (OPA) fluorescence assay, crystal violet assay, Total Organic Carbon (TOC), and CFUs. They were compared for sensitivity and fitness to assess biofilm reduction per ISO 15883 requirements. Although ISO 15883 requires that 5 cm length tubes be tested, in our study biofilms were quantified on tubes ranging in length from 5 cm to 0.5 cm in order to determine the potential of the five methods to accurately and precisely determine biofilm reductions from 50% to 90% (based on tube lengths, refer to material and methods section). The estimated reductions were compared to determine pairwise correlations and which methods were statistically equivalent.

2. Materials and methods

2.1. Biofilm model

Pseudomonas aeruginosa CIP A22 (Pasteur Research Institute, Paris, France) stock was streak plated on Tryptic Soy Agar (TSA) (Oxoid, Nepean, Canada) and incubated at 30 °C for 48 h. Colonies were then scraped and suspended in Trypticase Soy Broth (TSB) (Hardy Diagnostics, Santa Maria, USA).

Biofilm was grown using an endoscope lumen biofilm method as previously described [9]. *P. aeruginosa* was grown on the lumen surface of 2 m long, 4 mm internal diameter PTFE tubes, under laminar flow for 72 h at 30 °C. Following growth, the tube was gently rinsed with PBS (Sigma-Aldrich, St. Louis, USA). To evaluate each quantification method, samples were cut randomly at defined lengths to mimic a biofilm reduction when compared to a full (5 cm) sample. Indeed, this method allowed to build a standard curve of analyte quantity as function of the expected reduction. The tube was cut into 5 cm, 2.5 cm, 1.25 cm, 0.625 cm. and 0.5 cm portions, which allowed an assessment of whether each quantification method could estimate the target percent reductions (PR) of 50%, 75%, 87.5% and 90%, respectively, using the following formula.

$$\text{Target PR} = 100 \cdot (1 - [\text{length}/5 \text{ cm}])\%$$

During the five independent experiments, three samples of each size were cut at random positions from each 2 m tube and were quantified with every method.

2.2. Biofilm extraction

For methods requiring biofilm extraction (OPA, μBCA, TOC, and CFU), samples were cut in half, lengthwise to expose the lumen. Extraction was performed in Ultra Pure (UP) water produced by a MilliQ Academic water filtration system (Millipore, Burlington, USA). Both halves of each sample were transferred in 10 ml sterile Ultra Pure water containing approximately 3 g of 0.5 mm glass beads in 50 ml centrifugation tubes. Biofilm was extracted by vortexing the tubes for 3 min using a VX-2500 multi-tube vortexer (VWR, Radnor, USA) at full speed, ensuring the proper formation of a vortex in every extraction tube.

2.3. Biomass quantification by crystal violet

Biofilm contaminated tubes were submerged in a 0.01% crystal violet (BD, Mississauga, CA) solution for 5 min at room temperature. The tubes were rinsed in successive UP water baths until the excess dye was removed. The samples were then dried for 10 min at room temperature and transferred to 10 mL 75% ethanol with 2 g 0.1 mm glass beads and vortexed for 3 min to remove the biofilm from the tubing and dissolve the dye. A sterile, 5 cm PTFE tube sample was subjected to the staining process and was used as a blank control. Two hundred microliters of each sample were then transferred to a 96 well plate in triplicate and absorbance was read at 550 nm using a BioTek Cytation5 microplate reader (Agilent, Santa Clara, USA). Blank controls were subtracted from biofilm samples data prior to analysis. Absorbance results were then transformed in quantitative values expressed in μg/mL of crystal violet, using serial dilutions of crystal violet as a standard curve [11].

2.4. Colony forming unit determination

The extracted samples were diluted by 10-fold serial dilution in peptone water (3 M, St. Paul, USA). One hundred microliters of the targeted dilutions of each sample were plated in triplicate on TSA agar and incubated at 37 °C overnight. Following incubation, plates were enumerated and results were reported as CFU/mL prior to data transformation to CFU/samples.

2.5. Protein quantification by micro BCA

High concentration samples (5 cm tubes and 2.5 cm tubes) were diluted in sterile UP water to 1/10 and 1/5 ratio, respectively, in addition to quantifying the undiluted samples.

Protein quantification was performed using the Pierce micro BCA assay kit (Fisher Scientific, Rockford, USA). The standard curve was modified slightly in order to obtain a linear trend of the signal between 0 and 30 μg/mL. One milliliter of each sample and 1 mL of reactive solution was placed into a 96-wells deep well plate. The plate was covered and incubated at 60 °C for 60 min. The plate was then cooled to room temperature and 200 μL of each sample well were transferred in triplicate into a 96 well plate before measuring absorbance at 562 nm using the BioTek Cytation5 microplate reader.

2.6. Protein quantification by orthophtalaldehyde fluorescence assay

High concentration samples (5 cm tubes and 2.5 cm tubes) were diluted in sterile UP water to 1/10 and 1/5 ratio, respectively, in addition to quantifying the undiluted samples.

OPA reagent was prepared using the following component ratios: 0.04 g of orthophtalaldehyde (Laboratoire MAT, Québec, Canada), 1 mL of methanol, 0.1 g of N,N dimethyl-2-mercaptothylammonium chloride, 50 mL borax, and 1.25 mL sodium dodecyl sulfate. Calibration standards were prepared by diluting bovine serum albumin in sterile ultrapure water to obtain a linear trend of the signal between 0 and 16 μg/mL. Reaction samples were prepared by adding 0.1 mL of 20 % SDS and 1.9 mL of OPA reagent to 2 mL of each sample. Sample fluorescence was

measured in quartz cuvettes with a Cary Eclipse Fluorometer (Agilent) at an excitation wavelength of 338 nm, emission was captured at 425 nm with excitation and emission slits set at 5 nm, and signal acquisition time of 1 s.

2.7. Total Organic Carbon

Samples were diluted in sterile UP water as follows: 5 cm tubes samples were diluted to a 1/20 ratio, 2.5 cm tubes samples were diluted to a 1/10 ratio, all other samples, including blanks, were diluted to a 1/4 ratio. Total organic carbon was quantified using a GE Sievers 900 series TOC analyzer (General Electric, Boston, USA).

2.8. Data transformation

All blank-corrected data were converted in quantity of analyte per sample using the dilution factors applied during extraction and quantification. Crystal Violet, OPA, μ BCA, and TOC results were recorded as $\mu\text{g}/\text{mL}$ which were then converted to $\mu\text{g}/\text{sample}$ by multiplying by 10 after subtracting blank values. CFU values were multiplied by the dilution factor to determine the CFU/sample. All 5 responses from all tube sizes were \log_{10} -transformed, from which an observed log reduction (LR) for each response was then calculated using the following formula:

$$LR = \log_{10}(\text{response for 5 cm tube}) - \log_{10}(\text{response})$$

2.9. Statistical analysis

Target LRs were calculated based on the tube sizes using the following formula:

$$\text{Target LR} = \log_{10}(5 \text{ cm}) - \log_{10}(\text{tube size})$$

For the tube sizes 2.5, 1.25, 0.625 and 0.5 cm that we considered, the target LRs were 0.3, 0.6, 0.9 and 1, respectively. For each response, two different statistical tests were conducted to compare the observed LRs with the target LRs. 1) a linear mixed effects model (LMM) was fit to the difference in LRs (observed – target) with a fixed effect for tube size and random effects for replicate tube; 2) a LMM was fit to the log-transformed responses for the 0.5 cm and 5 cm tube sizes only with a fixed effect for tube size and random effect for replicate tube. The random effects were important because they account for the repeated measures (*i.e.*, the multiple tube samples of different lengths) collected from the same 2 m replicate tube. The inclusion of the interaction was important because the ranking of the tube sizes varied among the different replicate tubes. Statistical equivalence tests [12] were performed based on 95% two-sided Tukey confidence intervals (CIs) of differences of the mean LRs observed from the theoretical LR using an equivalency margin $0.5 \log_{10}$. The LMMs were fit using the statistical software Minitab v.20. Unusual data and the assumptions of the LMM were assessed with residual plots.

3. Results

Biofilms formed in PTFE tubes were quantified using five methods.

Table 1
Average quantification of the biofilm samples for the five replicates.

Sample size (cm)	5		2.5		1.25		0.625		0.5	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Log CFU	8.9	± 0.3	8.4	± 0.3	7.9	± 0.3	7.2	± 0.4	7.2	± 0.4
Protein OPA (μg)	239.6	± 71.5	119.6	± 20.6	60.6	± 8.7	39.1	± 12.6	31.8	± 8.3
Protein μ BCA (μg)	224.6	± 54.0	105.7	± 27.5	46.5	± 16.3	29.4	± 11.1	23.4	± 10.9
Total Organic Carbon (μg)	150.8	± 28.7	76.5	± 13.3	30.6	± 5.5	15.6	± 2.9	11.9	± 3.2
Crystal Violet (μg)	15.2	± 3.4	7.5	± 2.4	2.8	± 0.9	1.0	± 0.6	0.1	± 0.6

Table 1 shows the averages and standard deviations of the quantitative outcomes for each tube size across the five replicates. The 5 cm tubes contained $239.6 \pm 71.5 \mu\text{g}$ of protein when dosed using the OPA fluorescence assay, and $224.6 \pm 54.0 \mu\text{g}$ of protein when quantified with μ BCA assay. Total organic carbon quantification yielded $150.8 \pm 28.7 \mu\text{g}$, and $15.2 \pm 3.4 \mu\text{g}$ of crystal violet was bound to the biofilm. In addition, an average of $8.9 \pm 0.3 \log_{10}$ CFU were obtained from the 5 cm samples.

The observed LRs were plotted as a function of the target LRs in **Fig. 1**. The gray line in each pane is the line of equality. Hence, for an accurate method, the observed LRs should follow the gray line of equality. The OPA, μ BCA and TOC quantification methods followed the line of equality, with low LRs (≤ 0.6) closer and high LRs (> 0.6) further from the line of equality (more precise) whereas μ BCA LRs were more variable (less precise). CFU and CV LRs were generally above the equality line (biased high instead of accurate) and highly variable (not precise).

Statistical analysis of the LRs, presented in **Table 2**, was used to quantitatively assess the observations from **Fig. 1** regarding accuracy and precision of the 5 methods. Accuracy and precision were assessed by testing for equivalence of the observed and target mean LRs. The observed mean LRs for μ BCA, TOC and OPA were statistically equivalent to the target LRs because they were very close to the target LR (with a difference of $-0.023 \log_{10}$, $0.068 \log_{10}$ and $-0.083 \log_{10}$ respectively) with narrow 95% CIs around this difference (*i.e.*, the confidence limits are less than the equivalency margin of $0.5 \log_{10}$) (**Table 2**). The $0.5 \log_{10}$ equivalency margin was chosen based on its use by the US FDA, US EPA, and ASTM, as well as previously published examples [13–16]. The mean LR for crystal violet was $0.294 \log_{10}$ higher although still equivalent with the target. On average, CFU LRs were $0.555 \log_{10}$ higher than the target, which were clearly not equivalent with the target LRs. Finally, focusing on target LR = 1 by comparing responses from the 0.5 cm and 5 cm tube sizes allowed the accuracy and precision of the methods to be directly evaluated at the 90% reduction that was the suggested performance by the ISO 15883 standard. It was found that the μ BCA, TOC and OPA quantification of the 0.5 cm sample were statistically equivalent to the target of 1 (0.008 , 0.11 and $-0.13 \log_{10}$ different from the target respectively with narrow CIs). CFU and crystal violet LRs were not statistically equivalent to the target LR of 1, being 0.71 and $0.56 \log_{10}$ above target, respectively.

The \log_{10} -transformed responses for each quantification method were compared to each other, as well as to the size of the \log_{10} -transformed tube sample, using pairwise correlations. **Table 3** shows the correlation scores (*r* values) for the \log_{10} -transformed responses. TOC had the best association with the size of the tube sample ($r = 0.979$), a proxy for the target LR or PR, followed by OPA ($r = 0.951$), μ BCA ($r = 0.941$), and crystal violet ($r = 0.934$). CFU were the least correlated to the size of the sample tube ($r = 0.884$). When the 5 methods were compared pairwise, TOC and OPA were the most correlated methods ($r = 0.951$), followed by OPA and μ BCA ($r = 0.939$), TOC and μ BCA ($r = 0.917$). To a lesser extent, crystal violet responses were correlated to TOC ($r = 0.906$), μ BCA ($r = 0.870$) and OPA ($r = 0.861$). Finally, CFU were the least correlated to μ BCA ($r = 0.878$), OPA ($r = 0.859$), TOC ($r = 0.854$), and crystal violet ($r = 0.806$).

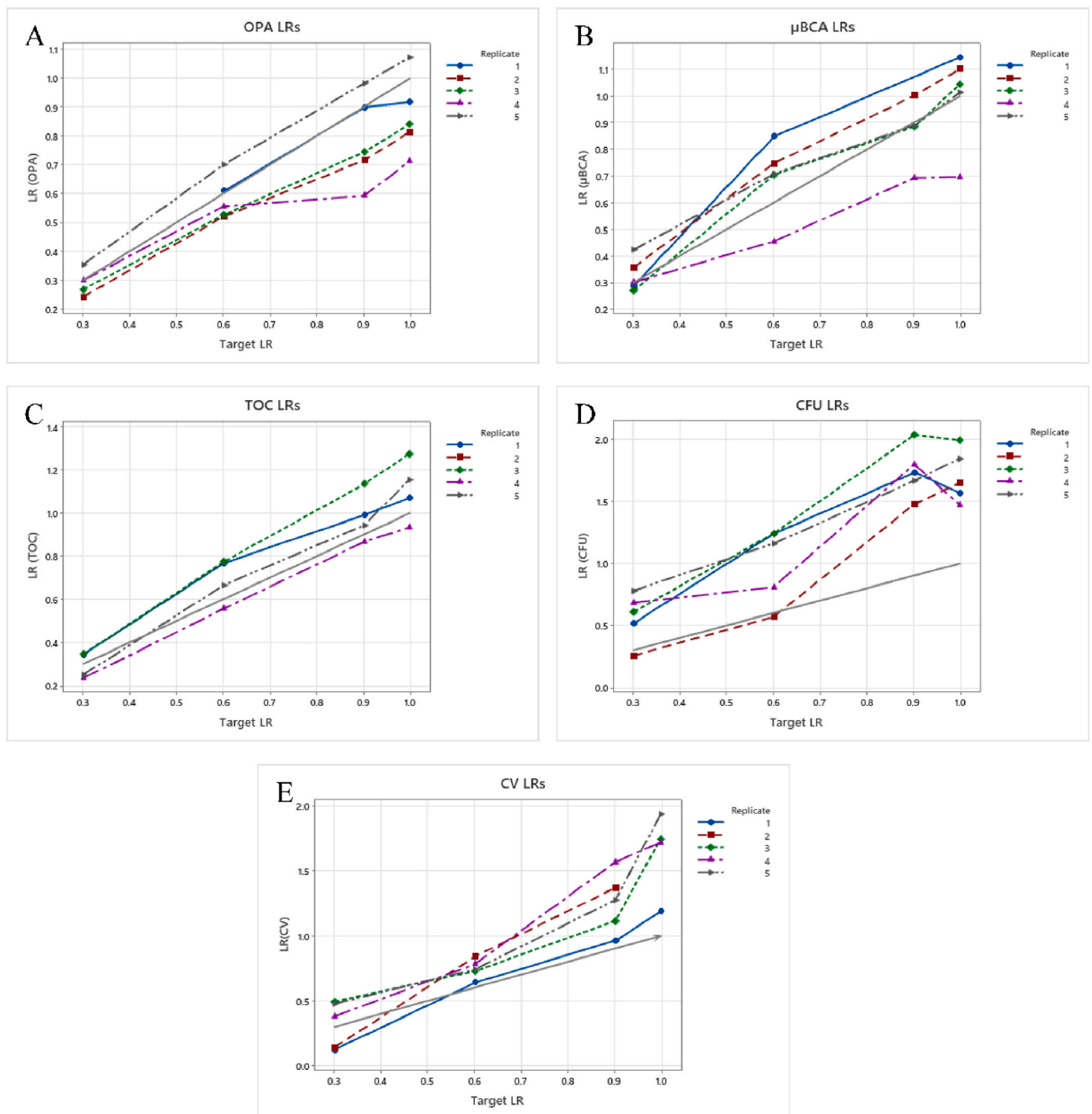


Fig. 1. Plots of the observed LRs versus the target LRs of 0.3, 0.6, 0.9 and 1 for each quantification method: A. OPA; B. μBCA; C. TOC; D. CFU; E. CV. The gray line in each pane indicates the line of equality. The non-gray colored lines indicate the results from each of 5 independent replicate experiments.

4. Discussion

Biofilm persistence in endoscope lumens has been linked to hospital acquired infections [2,4]. To mitigate the risk of HAIs in the future, automated endoscope reprocessor manufacturers need standard methods to assess biofilm removal. The only standard method available to grow and quantify biofilm cleaning in endoscope lumens is contained in ISO 15883-5 [6,9]. The resulting biofilm was demonstrated as clinically relevant and yielded higher protein content and CFUs than an endoscope lumen soiled after real use. The previous ISO 15883-5 technical specification Annex F detailed the method to perform testing

[17]. A key aspect in biofilm removal assessment is the quantification method used. Three analytes are proposed in the ISO technical specification annex when testing self disinfection of AER lumens: protein, carbohydrate, and CFU. When assessing cleaning of endoscopes lumen, only protein or carbohydrates are suggested. This study used the biofilm test soil model, following ISO TS 15883-5:2021 procedure for extraction [17], with a few deviations: instead of vortexing samples with sand in Ringer’s 1/4th buffer to extract biofilm, biofilm was extracted by vortexing in sterile water with glass beads. This extraction method has been internally verified to be equivalent to the original method (Data not shown). Indeed, it was found that sand particles interact with some of

Table 2

Statistical analyses of accuracy and precision of the quantification methods. Gold cells suggest accuracy and precision for methods where the observed LRs and the target LRs are equivalent across all tube sizes using an equivalency margin of $0.5 \log_{10}$. Blue cells suggest accuracy and precision for methods where the observed LRs and the target LR = 1 are equivalent for Tube Size = 0.5 cm using an equivalency margin of $0.5 \log_{10}$.

LR response	Num. Reps	Num. Samples	Testing of equivalence across all Tube Sizes $\Delta = \text{observed} - \text{target}^1$		Testing of equivalence at Tube Size = 0.5cm $\Delta = \text{observed} - \text{target}$	
			mean	95% CI	mean	95% CI
OPA	5	72	-0.083	[-0.198, 0.032]	-0.13	[-0.20, -0.05]
μ BCA	5	71	-0.023	[-0.169, 0.124]	0.008	[-0.09, 0.09]
TOC	4	60	0.068	[-0.087, 0.223]	0.11	[0.02, 0.20]
CFU	5	75	0.555	[0.328, 0.782]	0.71	[0.50, 0.92]
CV	5	67	0.294	[0.098, 0.489]	0.56	[0.36, 0.75]

1: The mean difference between the observed LR and the target LR is the mean difference between the colored lines and the gray lines of equality in Fig. 1.

Table 3

Pairwise correlations of the \log_{10} -transformed responses between quantification methods. The green box highlights the relationship between $\log(\text{OPA})$ and $\log(\text{TOC})$ that had the highest correlation among all pairs of responses ($r = 0.951$, $R^2 = 90.4\%$). The purple box highlights that TOC has best association with Tube Size ($r = 0.979$, $R^2 = 95.8\%$).

	$\log_{10}(\text{Tube Size})$	$\log_{10}(\text{OPA})$	$\log_{10}(\mu\text{BCA})$	$\log_{10}(\text{TOC})$	$\log_{10}(\text{CFU})$
$\log_{10}(\text{OPA})$	0.951				
$\log_{10}(\mu\text{BCA})$	0.941	0.939			
$\log_{10}(\text{TOC})$	0.979	0.951	0.917		
$\log_{10}(\text{CFU})$	0.884	0.859	0.878	0.854	
$\log_{10}(\text{CV})$	0.934	0.861	0.870	0.906	0.806

the quantification methods used in this study, especially protein quantification by the OPA fluorescence assay.

Additionally, the study relies on the assumption that the biofilm is homogeneous along the 2 m length of the lumen. This was evaluated prior to this study by staining sample tubes from random locations along the full 2-m length after contamination with biofilm grown according to the ISO 15883-5 Biofilm Test Soil model. Visual observation showed that the random sample tubes were stained homogeneously (see Supplementary Materials Fig. S1 for examples of stained samples). In addition, after our study was conducted, the average and SD of the 3 \log -transformed responses from each of the 5 sample length (0.5–5 cm) for each replicate were calculated. The coefficient of variation (SD/mean) was less than 17.4% for OPA, μ BCA, TOC and CFU (Supplementary Table S1), further supporting that the biofilm was homogeneous across the samples taken from random locations along the 2 m tube. For CV absorbance however, the coefficient of variation was as large as 300%, i.e., the CV SD was 3 times larger than the CV mean for one of the 5 replicate 2 m tubes, which suggests that CV is not a preferred response when measuring biofilm in this endoscope model. A key aspect of obtaining a homogeneous biofilm with this model is to ensure the absence of air bubbles in the tube during growth. Indeed, air disrupts the laminar flow in the tube during growth and prevents bacteria to deposit in air pockets adhered to the surface, resulting in a heterogeneous biofilm. When developing a standard biofilm model, homogeneity of the biofilm must be considered as a critical assumption.

The goal of this study was to assess five quantification methods easily accessible to the medical device industry's analytical laboratories: CFU, protein using the μ BCA assay kit, protein using a fluorescence OPA kit,

TOC, and CV. To do so, a standard curve was created using samples of decreasing sizes because controlling the removal of biofilm is not possible by direct exposure of the samples to an AER process. Alternatives to regulate biofilm removal were considered, such as manually scraping a specific surface area of the lumen or performing serial dilutions of 5-cm samples. However, scraping alone may not be sufficient to remove exactly the amount of biofilm. Dilutions of the extraction of a 5-cm sample could also have been performed, however introduction of variability to the results by the dilution process was anticipated. Therefore, it was determined that, in the absence of a gold standard in biofilm removal assessment, quantifying various lengths of the contaminated tubing was the most suitable way to establish the standard curves to evaluate the quantification methods.

Enumeration of CFUs has been the gold standard for biofilm quantification for decades. However, CFU counts only provide a partial assessment of biofilm quantification. It quantifies the living cells embedded in the biofilm matrix and allows a log reduction of these living cells to be calculated when compared to a positive control. However, CFU quantification cannot differentiate between viable but non-culturable cells and dead cells [18]. When assessing biofilm cleaning, the biofilm must be considered as a soil containing living cells. Therefore, CFU alone is not enough to assess biofilm cleaning or removal. In addition, this study demonstrated that CFU was highly variable and overestimated the target log reductions more than any other method.

Biofilms harbor protein in the matrix and the bacterial cells themselves also contribute to the total protein content. Two protein quantification methods were explored in this study. OPA, a method commonly

used for medical devices cleaning assessment as suggested in ISO 15883-5 [6], provided high sensitivity and low variability although the preparation is more complex than μ BCA. Alternatively, μ BCA requires little time to execute and can be found in commercially available kits. The two protein quantification methods showed high correlation with target log reduction values. Additionally, they had appropriate limits of detection to allow biofilm removal assessment according to ISO 15883 series criteria (90% reduction). Both methods also provided precise and accurate LRs with differences less than 0.1 logs from the target LR. OPA was able to differentiate between tube sizes with a negligible underestimate of the target LR. Micro BCA was also able to differentiate between tube size results and the data most closely matched the estimated target LR. Since differences between observed LR and target LR were negligible with both methods, they can be considered equivalent methods. The two methods used to quantify protein (OPA and μ BCA) could have been compared using absolute quantification responses (*i.e.* the amount of protein). However, due to the differences inherent to the methods, a shift in absolute quantification response was observed between the two methods. In the OPA method, the reaction works by binding of the OPA to the α -amino acid (N-terminus) [19] of proteins whereas with the μ BCA method, the reagent reacts with the copper ions originating from the biuret reaction [20]. The amount of copper ions depends on the presence of cysteine, tyrosine and tryptophan side chains. These components reacting differently could explain this shift in quantification.

Crystal violet has a long history of being used to quantify biofilm in research. The binding properties of CV to peptidoglycan and to polysaccharides allow to stain the biofilm and quantify the biomass [21,22]. However, in most studies, absorbance measurements provide a relative quantification not an absolute quantification. In their recent study, Allkja et al. established a standard curve for CV to allow quantification in terms of biomass equivalent to μ g of CV bond [11]. In this study, a similar approach was used to allow absolute quantification. It was found that CV was unable to accurately quantify biofilm at the lowest tube size (*i.e.*, lowest target log reductions) and consistently overestimated the LR in biofilm therefore risking false pass results in a biofilm cleaning assessment. In addition, the coefficient of variation of the CV response was as high as 300%, much higher than the other responses, and thus the most variable response (Supplementary Table S1). Nonetheless, it remains useful as a rapid method to visually confirm biofilm removal from a PTFE surface.

Total organic carbon is a marker used in the pharmaceutical industry for cleaning validation [23]. It is also a common analyte used for cleaning assessment of medical devices per ISO 15883-5 standard [6]. The assay determines the concentration of organic compounds in a solution. It is a promiscuous method that cannot differentiate between living organisms and other organic matter. In this study TOC provided the most accurate and precise results when differentiating between tube sizes, with only a negligible overestimate of the biofilm log reduction.

Therefore, this study suggests that TOC and protein are appropriate analytes to be used in the quantification of biofilm reduction when assessing biofilm removal using the biofilm test soil model designed by Pineau et al. [9]. When information regarding disinfection level is required, CFU should be considered as an additional analyte.

The biofilm test soil model is recommended by ISO 15883-5 as test soil for lumen cleaning performance of automated endoscope reprocessors using *P. aeruginosa*. However, when first published, the method also allowed monospecies biofilm formation with similar results using *E. coli* or *S. aureus* [9]. In the past decades, interest for multispecies biofilm has increased. The model presented in the current study is a single species biofilm, as recommended by the ISO 15883 standard series [6]. Perspectives to update this method using a representative multi-species biofilm could be considered in order to improve the model. It has indeed been previously shown that multispecies biofilms present a greater challenge for cleaning and disinfection [24,25]. Therefore, when developing a biofilm method, multispecies biofilms should be considered when applicable.

5. Conclusion

CFU alone should not be used to assess biofilm cleaning or removal since it only quantifies the living aspect of the soil and not remaining matrix or dead cells. Crystal violet allowed rapid and easy assessment of the reduction but was neither the most accurate nor the most precise method. TOC and protein (both μ BCA and OPA methods) showed high correlation to the biofilm reduction with both adequate accuracy and precision. Therefore, this study suggests that biofilm removal can be quantified using protein or TOC. Also, it provides a strategy to statistically assess the validity of quantification methods for assessing biofilm removal. However, the compatibility of these quantification methods must be assessed with different biofilm models. With the technological advances in the medical devices industry and the biofilm concern in reusable medical devices, there is opportunity and dire need for regulatory science research to continue finding appropriate models and methods to assess biofilm cleaning and disinfection.

Declarations

The study was funded by STERIS. STERIS is a manufacturer of Automated Endoscope Reprocessors.

Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Bruno Haas reports a relationship with STERIS that includes: employment. Marie-Claude Gagnon reports a relationship with STERIS that includes: employment. Sarah James reports a relationship with STERIS that includes: employment. Philippe Labrie reports a relationship with STERIS that includes: employment. Noemie Goulet reports a relationship with STERIS that includes: employment. BH, SJ, MCG, NG, and PL are employees of STERIS, an Automated Endoscope Reprocessors manufacturer.

CRediT authorship contribution statement

Bruno Haas: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Sarah James:** Investigation, Formal analysis, Writing – original draft, Visualization, Validation. **Albert E. Parker:** Formal analysis, Writing – review & editing, Visualization. **Marie-Claude Gagnon:** Resources, Investigation, Writing – review & editing, Methodology, Validation. **Noémie Goulet:** Resources, Investigation, Validation. **Philippe Labrie:** Conceptualization, Supervision, Project administration, Writing – review & editing.

Data availability

The data that has been used is confidential.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biofilm.2023.100163>.

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