American Journal of Infection Control 46 (2018) 159-64

Contents lists available at ScienceDirect

American Journal of Infection Control

journal homepage: www.ajicjournal.org

Major Article

Potential testing of reprocessing procedures by real-time polymerase chain reaction: A multicenter study of colonoscopy devices

Federica Valeriani PhD [a], Antonella Agodi PhD [b], Beatrice Casini PhD [c], Maria Luisa Cristina PhD [d], Marcello Mario D'Errico MD [e], Gianluca Gianfranceschi BS [a], Giorgio Liguori PhD [f], Renato Liguori PhD [f], Nicolina Mucci PhD [g], Ida Mura MD [h], Cesira Pasquarella MD [i], Andrea Piana PhD [h], Giovanni Sotgiu MD [h], Gaetano Privitera MD [c], Carmela Protano MD [j], Annalisa Quattrocchi PhD [b], Giancarlo Ripabelli PhD [k], Angelo Rossini MD [l], Anna Maria Spagnolo PhD [d], Manuela Tamburro PhD [k], Stefano Tardivo MD [m], Licia Veronesi MD [i], Matteo Vitali PhD [j], Vincenzo Romano Spica MD [a,*], GISIO Working Group of the Italian Society of Hygiene, Preventive Medicine, and Public Health

[a] *Department of Movement, Human and Health Science, University of Rome "Foro Italico", Rome, Italy*
[b] *Department of Medical and Surgical Sciences and Advanced Technologies "GF Ingrassia," University of Catania, Catania, Italy*
[c] *Department of Translational Research and New Technologies in Medicine and Surgery, Pisa University, Pisa, Italy*
[d] *Department of Health Sciences, University of Genoa, Genoa, Italy*
[e] *Department of Biomedical Sciences and Public Health, Politechnic University of Marche, Ancona, Italy*
[f] *Department of Movement and Health Sciences, University "Parthenope," Napoli, Italy*
[g] *Department of Technological Innovations and Safety of Plants, Products and Anthropic Settlements, National Institute for Insurance against Accidents at Work, INAIL, Rome, Italy*
[h] *Department of Biomedical Science—Hygiene Section, University of Sassari, Sassari, Italy*
[i] *Department of Medicine and Surgery, University of Parma, Parma, Italy*
[j] *Department of Public Health and Infectious Diseases, Sapienza University of Rome, Rome, Italy*
[k] *Department of Medicine and Health Sciences "Vincenzo Tiberio," University of Molise, Campobasso, Italy*
[l] *Fondazione Santa Lucia Institute for Research and Health Care, IRCCS, Rome, Italy*
[m] *Department of Public Health and Community Medicine, University of Verona, Verona, Italy*

Key Words:
Health care-associated infections
Flocked swab sampling
mfDNA
Sanitation
Surveillance

Background: Reprocessing of endoscopes is key to preventing cross-infection after colonoscopy. Culture-based methods are recommended for monitoring, but alternative and rapid approaches are needed to improve surveillance and reduce turnover times. A molecular strategy based on detection of residual traces from gut microbiota was developed and tested using a multicenter survey.
Methods: A simplified sampling and DNA extraction protocol using nylon-tipped flocked swabs was optimized. A multiplex real-time polymerase chain reaction (PCR) test was developed that targeted 6 bacteria genes that were amplified in 3 mixes. The method was validated by interlaboratory tests involving 5 reference laboratories. Colonoscopy devices (n = 111) were sampled in 10 Italian hospitals. Culture-based microbiology and metagenomic tests were performed to verify PCR data.
Results: The sampling method was easily applied in all 10 endoscopy units and the optimized DNA extraction and amplification protocol was successfully performed by all of the involved laboratories. This PCR-based method allowed identification of both contaminated (n = 59) and fully reprocessed endoscopes (n = 52) with high sensibility (98%) and specificity (98%), within 3-4 hours, in contrast to the 24-72 hours needed for a classic microbiology test. Results were confirmed by next-generation sequencing and classic microbiology.

* Address correspondence to Vincenzo Romano Spica, MD, Department of Movement, Human and Health Science, University of Rome "Foro Italico," Piazza Lauro De Bosis 6, 00135 Rome, Italy.
E-mail address: vincenzo.romanospica@uniroma4.it (V. Romano Spica).
Conflicts of interest: None to report.
Funding for this study was provided by the Italian Study Group of Hospital Hygiene (GISIO), National Working Group of the Italian Society of Hygiene, Preventive Medicine, and Public Health.
The reagents used in this study were provided by MDD University Spin Off, Viterbo, Italy, and Copan Italia, Brescia, Italy.

0196-6553/© 2018 Association for Professionals in Infection Control and Epidemiology, Inc. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
https://doi.org/10.1016/j.ajic.2017.08.008

Conclusions: A novel approach for monitoring reprocessing of colonoscopy devices was developed and successfully applied in a multicenter survey. The general principle of tracing biological fluids through microflora DNA amplification was successfully applied and may represent a promising approach for hospital hygiene.

© 2018 Association for Professionals in Infection Control and Epidemiology, Inc. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Endoscopy plays an essential role in diagnosis and therapy of several diseases.[1] This costly and sophisticated approach is based on reusable tools that need appropriate reprocessing to avoid cross-infections.[2] Because of the type of endoscopy, the cleaning treatments used, and patient clinical features, the disinfection or sterilization levels may vary in their efficacy requirements.[3,4] Failures during reprocessing steps may occur, and contaminated endoscopes have been associated with outbreaks of health care-associated infections more frequently than any other medical devices.[2,5] Remarkably, endoscope-transmitted infections can occur even when reprocessing is performed following professional and manufacturer guidelines.[5-7] Flexible endoscopes are significantly contaminated by biological fluids, including blood or secretions.[6-8] Radical cleaning is a critical step due to the complex structure of the devices, which are characterized by narrow lumens and multiple internal channels. Endoscope reprocessing is a challenging task involving cleaning and high-level disinfection (HLD) treatments, followed by rinsing and drying before appropriate storage.[8-10] Reprocessing failures, as well as the ability of bacteria to form biofilm on the inner channels and surface roughness, increase the likelihood of health care-acquired infections.[2,11-13] Education-based actions and technical advancements can certainly improve reprocessing effectiveness and ensure proper safety levels through multidisciplinary teamwork.[2,12,14,15] Microbiologic surveillance by culture-based methods represents an established and easy-to-use approach to assess the effectiveness of reprocessing procedures, but relevant limitations should be considered, such as long response time, low specificity, and poor sensitivity, in detecting resistant microorganisms not cultivable on standard media such as viruses, protozoa, prions, or viable but not cultivable bacteria.[6,13,15-17]

Recently, rapid biochemical methods, based on the evaluation of ATP, protein, carbohydrate, or hemoglobin levels, have been proposed to assess removal of organic residues from endoscopes.[7,18,19] However, several shortcomings, such as aspecific output, low sensitivity, and interference with disinfectants, suggests further improvements are needed.[18,19] DNA-based techniques, including real-time polyerase chain reaction (PCR), may indeed show several advantages in comparison with traditional culture-based methods in that they are less time-consuming, highly specific and sensitive, affordable, and can detect viable but not cultivable bacteria.[20,21] The potential application of molecular techniques represents a very promising and challenging opportunity to further improve monitoring of reprocessed devices. Here, we report the use of a novel method to monitor the effectiveness of reprocessing by an optimized real-time PCR approach that was evaluated on colonoscopy devices using a multicenter survey. The general principle of the method was based on the observation that residual traces of biological fluid microflora (mf) on reprocessed devices represent a potential indicator of sanitation failure, when tested by an mfDNA-based approach.[22] The identification and characterization of biological fluid by mfDNA analysis were initially applied in forensics and then hospital hygiene in dental settings.[22,23] In this study, sampling and DNA extraction were developed and validated within interlaboratory tests to achieve a simple and rapid protocol for a routine monitoring. Next-generation sequencing (NGS) analysis on selected mfDNA samples was carried out to confirm the molecular data. The general principle of tracing biological fluids through mfDNA amplification was applied in a multicenter study, suggesting promising perspectives for surveillance.

MATERIALS AND METHODS

Setting and study design

This study was conducted in the main hospitals of different Italian regions (Campania, Emilia Romagna, Lazio, Liguria, Marche, Molise, Tuscany, Veneto, Sardinia, and Sicily) involving 10 endoscopy units that reprocess approximately 50-100 endoscopes per business day. Data were collected by the Coordinating Laboratory Unit in Rome (Lazio, Foro Italico). Following a checklist, each participant unit (PU) provided information on endoscopy devices and reprocessing procedures or locally available guidelines, department and referent identification, and documentation on the reusable instruments subjected to sanitization. Each PU received a kit containing nylon-tipped flocked swabs with drying active agent (4N6FLOQSwabs; Genetics, Copan Italia, Brescia, Italy) and information on sampling and storage procedures. Sampled specimens were anonymously coded and sent to 1 of 5 reference laboratories (RLs) located in different Italian regions (Tuscany, Sardinia, Sicily, Molise, and Lazio). Colonoscopy devices included in this study were Olympus (Lake Success, NY) or Pentax (Montvale, NJ) and all underwent precleaning and manual cleaning procedures according to the endoscope manufacturer's instructions.[24,25] Briefly, the HLD was performed by automated endoscope reprocessors: CISA (ERS, Milan, Italy), Olympus, Medivators (Minntech, MN), and Pentax. After HLD, all endoscope channels were rinsed and forced air was used to dry the channels. Each RL performed DNA extraction and amplification as well as sampling and processing of their own samples. In each RL, 1 operator was responsible for all DNA extractions and real-time PCR. The RL received all supplementary materials for processing the samples, including DNA extraction kits (QIAmp DNA Mini Kit and DNeasy Blood & Tissue Kit; Qiagen, Hilden, Germany), NAO (nucleic acids optimizer) basket (Copan Italia), lysozyme solution and glass beads, 1 spiked sample as an internal positive control, the Microsan-F Kit (MDD University Spin Off, Viterbo, Italy) containing standardized amplification mix with aliquots of positive and negative controls, and the protocol. All kits and reagents were from the same batches.

Sampling

Each PU identified and sampled at least 10 colonoscopy devices: 5 dirty and 5 clean. For sampling, each nylon-tipped flocked swab was wiped on a surface area of 5 cm^2 (initially, inner channel sampling was considered, showing similar results, but in this preliminary phase of the study the simplest and most reproducible approach was selected, applied, and reported). Samples were collected using aseptic techniques in a dedicated room. In addition to routine monitoring, randomly selected additional samples (n = 40) were also collected to be analyzed by classic microbiology, following Centers for Disease Control and Prevention and European Society of Gastrointestinal Endoscopy and European Society of Gastroenterology and Endoscopy Nurses and Associates (ESGE-ESGENA) protocols.[16,26]

Briefly, the swabs were vortexed for 5 minutes in 10 mL sterile phosphate buffered saline and an aliquot of 1 mL was inoculated onto tryptic soy agar (total microbiology count), violet red bile dextrose agar (Enterobacteriaceae), and Baird-Parker agar (staphylococci) and plates were incubated aerobically at 37°C for 24-48 hours. Pure cultures were presumptively identified based on their morphology, colonial, and Gram staining characteristics. Their identity was confirmed by the oxidase, catalase, coagulase, motility, growth on Kliger iron agar, and the analytical profile index 20E assay (BioMerieux SA, Mercy l'Etoille, France).

Optimization of DNA extraction protocol and interlaboratory validation test

To simplify the extraction protocol, preliminary tests were performed and 2 different approaches for DNA extraction were considered, using traditional cotton swabs (GMBH, Germany) and nylon-tipped flocked swabs with active drying agent (Copan Italia). The comparison of the protocols and the optimization tests were performed preparing mock contaminated swabs (about 10^4 CFU), using a solution from fecal samples or a pure culture of *Enterococcus faecalis* ATCC 7080.

Protocol 1 has been described elsewhere,[23] but appeared too labor- and time-consuming for routine surveillance. Briefly, each swab was washed in 500 µL sterile phosphate buffered saline buffer (AppliChem GmbH, Darmstadt, Germany) at room temperature in agitation for 45 minutes. After the removal of the swab, the buffer was centrifuged at 16,000 × gram and the bacteria pellet was frozen at –20°C. After adding the glass beads (Sigma-Aldrich, St Louis, MO) the pellet was manually disaggregated with a pestle and lysed in 200 µL lysozyme solution, RNaseA treated, and proteinase K digested according to the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). Finally, DNA elution was performed in 50 µL elution solution (10 mM tris(hydroxymethyl)aminomethane-hydrochloride and 0.5 mM ethylenediaminetetraacetic acid, pH 9.0).

Protocol 2 was set up to provide a more rapid and transferable procedure in hospital surveillance settings. Briefly, each swab was extracted using the QIAamp DNA Mini (QIAmp DNA Mini Kit and DNeasy Blood & Tissue Kit) according to the DNA purification from buccal swabs procedure with minor modifications, and using the NAO Baskets. After sample collection, each swab was inserted into the semipermeable NAO Baskets and was broken inside at the breakpoint. Approximately 200 µL lysozyme solution (20 mg/mL Lisozima [Sigma-Aldrich], 20 mM tris[hydroxymethyl]aminomethane-hydrochloride at pH 8, 2 mM ethylenediaminetetraacetic acid, and 1.2% Triton X-100 [Sigma-Aldrich]) were added into the NAO Baskets and incubated a 37°C for 30 minutes. Then, 20 µL proteinase K and 400 µL buffer AL were added and the sample was centrifuged at 10,000 × g for 1 minute, allowing the elution of the digestion solution. After incubation at 56°C for 10 minutes and addition of 400 µL ethanol, the washing step and DNA purification were performed in accordance with the manufacturer's instructions. DNA elution was completed in 60 µL elution solution (10 mM tris[hydroxymethyl]aminomethane-hydrochloride and 0.5 mM ethylenediaminetetraacetic acid at pH 9.0).

Preparation of spiked samples for interlaboratory validation

The 5 RLs participated in an internal survey to validate the adopted approach. Each RL received in blind a spiked swab for DNA extraction and amplification. The spiked samples consisted of 100 µL fresh bacterial suspension of *E faecalis* corresponding to 10^5 CFU, spotted on nylon swabs and sent from the Coordinating Laboratory Unit to each RL. DNA was extracted according to protocol 2 and analyzed in parallel in all laboratories.

Analysis of mfDNA by multiplex real-time PCR and data interpretation

Amplifications were combined in 3 multiplex reactions (Microsan-F Kit; MDD, Viterbo, Italy): mix F1, for the identification of *Staphylococcus aureus*/*Enterococcus* spp; mix F2 for *Bacteroides fragilis*/*Bacteroides vulgatus*; mix F3 for *B vulgatus*/*Escherichia coli* (probes were labeled FAM/JOE/ROX, with the BHQ-1 quencher). Reactions were performed in a volume of 25 µL, of which 12.5 µL JumpStart Taq ReadyMix for Quantitative PCR (Sigma Aldrich, St. Louis, MO), containing 900 nM forward and reverse primers, and 250 nM of each probe. For each mix, samples were tested in triplicate.

The amplifications were performed using different instruments: ABI PRISM 7000 and 7900 HT (Life Technologies), Mastercycler (Eppendorf, Milan, Italy), Bio-Rad CFX96 (Bio-Rad, Hercules, CA) programmed for 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. For each sample 5-10 µL template reaction was amplified. The PCR output was expressed as cycle threshold (C_T), a measure of the quantity of detected DNA. Positive samples were considered those where C_T data analysis provided at least 1 positive indicator ($C_T \leq 33$) in at least 2 mixes. Conversely, a microbial indicator was considered negative when over the $C_T \geq 33$ threshold.

Statistical data analysis

The experimental tests were performed in triplicate and quantitative data were summarized using means and standard deviations. The performance of mfDNA analysis in the interlaboratory experimental study and in the multicenter survey was calculated in terms of sensitivity, specificity, false positive and false negative rates, efficiency, and selectivity, as follows: sensitivity: $a/(a+b)$; specificity: $d/(c+d)$; false positive rate: $c/(a+c)$; false negative rate: $b/(b+d)$; efficiency: $(a+d)/n$; and selectivity: $\text{Log}_{10}[(a+c)/(a+b+c+d)]$ where "a" is the number of true positives, "b" is the number of false positives, "c" is the number of false negatives, "d" is the number of true negatives, and "n" is the number of samples. False negatives were considered all those samples testing negative in used and/or not fully sanitized devices; false positives were all those samples testing positive in unused and/or fully sanitized devices.

NGS analysis

NGS samples were prepared according to the 16S Metagenomic Sequencing Library Preparation guide (part# 15044223 rev A; Illumina, San Diego, CA). The PCR amplicon was obtained using primers (containing overhang adapters) as previously described.[27,28] Libraries were quantified through PicoGreen dsDNA Quantitation Assay (ThermoFisher Scientific, Waltham, MA) and validated on Bioanalyzer DNA 1000 chip (Agilent, Santa Clara, CA) for the presence of the expected amplicon. Sequencing was performed on a MiSeq desktop sequencer (Illumina). The sequence reads were analyzed in the cloud environment BaseSpace through the 16S Metagenomics app (version 1.0.1; Illumina): the taxonomic database used was an Illumina-curated version of the May 2013 release of the Greengenes Consortium Database (greengenes.secondgenome.com); the classification algorithm was an implementation of the Ribosomal Database Project Classifier.[29] The Principal Coordinates Analysis was generated through the 16S Metagenomics app using classic multidimensional scaling on a Pearson covariance distance matrix created from per-sample normalized classification abundance vectors. Detection cutoff was set at 1%. NGS analysis was performed on a subset of positive samples ($n = 48$), whenever enough DNA was available for this additional experiment.

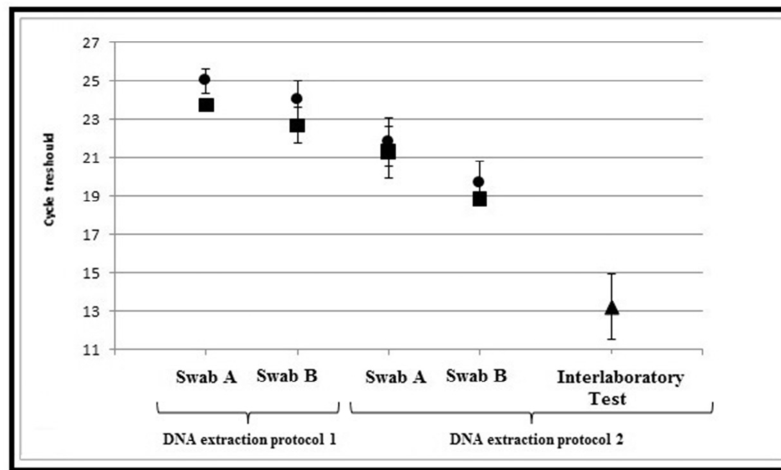

Fig 1. Optimization of sampling and DNA extraction. DNA recovery with extraction in protocol 1 and 2 using (A) cotton and (B) nylon swabs and final interlaboratory test using protocol 2 for DNA extraction. ●, fecal; ■, *Enterococcus faecalis*; ▲, validation sample.

RESULTS

Optimization of DNA extraction protocol and interlaboratory validation of the real-time PCR approach

The optimization of the DNA extraction protocol was required to simplify the monitoring procedure and acquire a more easily transferable and routinely acceptable protocol. It was successfully achieved, reducing the labor- and time-consuming steps; results are reported in Figure 1. Adequate yield of amplifiable DNA was always obtained (>20 ng/μL) from mock-contaminated swabs, allowing us to obtain C_T values in the range of 18.5-25.6. Protocol 2 using nylon flocked swabs was the most rapid (1.5 vs 3.5 hours) and effective (C_T of 19 vs 24) method, allowing amplification at a lower C_T number, with an higher sensibility corresponding to more than an additional log for DNA recovery ($\Delta C_T > 5$).[20] For this reason, we finally adopted protocol 2 and nylon flocked swabs to perform the interlaboratory test between the 5 RLs. All laboratories successfully applied the protocol, performing DNA extraction and real-time amplification of spiked samples and obtaining consistent results with C_T values ranging from 11.0-15.1 (mean, 13.2 ± 1.7). An additional interlaboratory test was performed comparing the amplification outputs obtained by amplifying the DNA standard controls using different PCR instruments. All laboratories identified and quantified correctly each target species within the different multiplex reactions, independently by the equipment, and providing a C_T within an acceptable range (mean, 18-28). The optimized protocol was easily and successfully applied in all laboratories and both DNA extraction and amplification steps were validated.

In-field multicenter survey

A total of 111 samples from colonoscopy devices, including used devices (n = 59) and fully reprocessed endoscopes (n = 52), were collected by the 10 PUs and processed by the 5 RLs. The results from the in-field multicenter survey are summarized in Table 1. Regarding the used devices, 98% resulted clearly positives and only 1 sample was not amplifiable by any indicator in any of the multiplex amplification mixes (sample 10 s). The majority of samples collected from fully reprocessed colonoscopy devices resulted in clear negatives (90.4%), whereas in 5 samples mfDNA traces were detected (at least 2 markers in at least 2 amplification mixes), suggesting a possible failure in reprocessing (samples 23p, 26p, 28p, 31p, and 43p).

Table 1
Multicentric survey summary. Testing of colonoscopy devices by the microflora DNA real-time polymerase chain reaction method

	Used	Reprocessed	Total
Total samples	59	52	111
Positive samples*	58	5	63
Negative samples	1	47	48

*At least 1 positive indicator (cycle threshold < 33) in at least 2 mixes.

No single microbial indicator could explain all the observed positive samples, but the combination of the different target genes for the selected microbial markers produced satisfying results. In particular, *B vulgatus* 16S amplicon accounted for 92.2% of positive results (59 out of 64), *B vulgatus* OmpA accounted for 70.3% (45 out of 64), *Enterococcus faecalis* accounted for 65.6% (42 out of 59), *Escherichia coli* accounted for 65.6% (42 out of 64), *B fragilis* accounted for 51.6% (33 out of 64), and *S aureus* accounted for 35.6% (23 out of 64). Whenever *B vulgatus* 16S amplicon was not detected, *B vulgatus* OmpA and/or *Enterococcus faecalis* were present, accounting for about 87% of samples (8 out of 59) from not-reprocessed endoscopes, and 80% of reprocessed endoscopes (1 out of 5) that were identified as positive. However, the simultaneous use of all 3 amplification mixes with the selected combination of primers provided an acceptable rate of positivity (>98%). Furthermore, internal negative and positive controls were always satisfied in all the assays performed by the RLs using the different instruments.

NGS analysis

To confirm the presence of the specific gut microbiota on the positive samples and exclude accidental contaminations or real-time PCR artifacts, samples identified as positive were tested by NGS, whenever sufficient DNA was available, for a total of 48 positive samples analyzed. Particularly, NGS analysis was performed on used/not-reprocessed devices (45 out of 58 [77.6%]) and fully reprocessed devices (3 out of 5 [60%]), showing a clear correspondence between real-time PCR data and gut origin (intestinal microbiota) of the mfDNA traces collected by swab sampling. The NGS analysis was valid, providing a sufficient total number of reads passing the quality filter (n = 5,835,693) and all samples presented at least 100,000 reads submitted for bioinformatic analysis.[30] Classification showed that

Table 2
Method performance. Experimental test performed in laboratory under controlled conditions and in field test based on the multicentric survey data

	Results	
Characteristic	Experimental test	In-field test
Sensitivity, %	100	98
Specificity, %	100	98
Efficiency, %	100	98
False positives	0	0.017
False negatives	0	0.02
Selectivity	−0.06	−0.30

Bacteroides genus was most represented (reads, 13.7%-68.2%; mean, 33.3% ± 15.3%) with the exception of some samples dominated by *Enterococcus* genus (6s, 22s, 39s, 41s, and 5s; mean, 83.5% ± 10.1%), in agreement with real-time PCR data. NGS analysis also confirmed that the fully reprocessed devices (31p, 28p, and 43p) were positive for mfDNA traces, suggesting possible failures in colonoscopy device reprocessing. In these samples, NGS allowed detection of the presence of additional fecal bacteria genus, typical of intestinal biologic fluid and gut microbiota, such as *Clostridium* and *Faecalibacterium*.

Evaluation of method performance

Data from interlaboratory tests and in-field surveys were used to evaluate the performance of the method, as reported in Table 2. In-field performance of the method was defined after confirming doubt samples by classic microbiology and/or NGS data. Interlaboratory assay using spike swabs provided the highest sensitivity and specificity values (100%), whereas slightly lower values (98%) were obtained by the in-field survey. Initially, raw in-field data provided acceptable but lower values for sensibility (92%). However, not all true-positive samples were confirmed by microbiology and/or NGS as clean and fully reprocessed samples. These apparently fully reprocessed colonoscopy devices that resulted in positive results by real-time PCR were further verified to exclude method failures or laboratory errors. In particular, sample 23p came back positive also using classic microbiology monitoring, so it was considered a true positive. Samples 28p, 31p, and 43p were confirmed positive by NGS analysis as showing presence of gut microbiota and therefore all were considered true positives. For sample 26p, additional DNA was not available for NGS, but because it was negative at microbiologic analysis, it was prudently considered a false positive. Based on this additional information, in-field performance values were updated as reported in Table 2, with a further increase in efficiency, from 95% to 98%. However, both performance evaluations—in lab and in field—support an acceptable performance for the tested real-time PCR method, with sensitivity, specificity, and efficiency between 92% and 100%.

DISCUSSION

Recent studies have reported the possible failure of endoscope reprocessing even when performed in accordance with professional and manufacturer guidelines.[5,7-12] Residual presence of organic materials and biological debris not fully removed during cleaning represents a risk for cross-infections and can reduce the HLD effectiveness.[3] Endoscopy-transmitted infections have been reported in different settings and appropriate microbiologic surveillance is strongly recommended by guidelines.[9-12] However, even a negative culture does not completely exclude the possibility of a contaminated device, as recently underlined by the Centers for Diseases Control and Prevention.[26] Therefore, the availability of alternative and rapid methods for monitoring reprocessing is strongly needed to overcome the limits of culture-based testing and speed up the endoscope turnover during routine clinical practice. Several innovative strategies have been introduced that show promising perspectives but also unsolved limitations, including the effectiveness of their performance in surveillance and compatibility with endoscope materials.[7,18,19] Some of these alternative methods have the advantage of being very rapid and simple, but they are less sensitive than DNA amplification. Moreover, the different chemical alternatives are not specific, providing generic information on the presence of organic contamination and not on its biological nature, source, or infectious risk.[18-21]

For the first time, to our knowledge, a DNA-based approach was applied to examine colonoscopy device reprocessing. A multiplex, real-time PCR protocol was designed to detect residuals of gut biological fluid as an indicator of reprocessing failure. The general principle is the identification of residual organic debris or biological fluid traces by the detection of marker genes belonging to the gut microbiota. Real-time PCR assays were developed targeting 6 genetic markers that resulted simultaneously in 3 independent reaction mixes. The assays allowed identification of positivity thresholds ($C_T < 33$) and the acquisition of a microbial signature typical of that biological fluid, providing rapid and specific information on the contamination source and its traceability.[28] Indeed, this strategy was effective in covering the naturally occurring microbial variability, as confirmed by the NGS data showing a clear presence of these and other species belonging to the intestinal microbiota.

Considering the ordinary complexity of surveillance needs in clinical settings, we tried to further simplify both sampling and DNA purification steps. A procedure based on nylon-tipped flocked swabs and a dedicated extraction protocol was successfully optimized to obtain a less labor- and time-consuming method that appeared efficient and suitable for both gram-negative and gram-positive bacteria. This protocol was validated in an interlaboratory test, showing high reproducibility, transferability, and an amplification at a lower C_T number, corresponding to an increased DNA recovery. Finally, with respect to traditional culture-based methods that require long incubation times (24-72 hours), this protocol allows faster monitoring of reprocessed devices with a potential influence on their turnover because the overall specimen analysis required about 3-4 hours, including both DNA extraction and amplification.

Once the protocol was established, it was applied for the multicenter survey, involving endoscopy units from 10 different major Italian hospitals. The sampling protocol was easily applied by all participants who followed the provided instructions. Likewise for the DNA analysis performed in 5 independent laboratories using different real-time PCR instruments. This confirms the high transferability of the method. Of sampled colonoscopies, 98.2% were correctly assigned by real-time PCR analysis and further confirmed by microbiology or NGS. The PCR assays allowed detection of 4 reprocessing failures, supporting the effectiveness of the strategy in endoscope surveillance and in monitoring local sanitation procedures. The overall performance of the method was 100% in the experimental interlaboratory testing and when calculated in the field during the multicenter survey.

The results of this multicenter survey support a promising applicability of the proposed method in the surveillance of reprocessed colonoscopy devices. Additional benefits are related to shorter turnover of endoscopy devices, whereas surveillance culture results would still be pending. However, limitations could be related to the DNA-based analysis. First of all, a molecular biology laboratory equipped with real-time PCR platforms is required and this may not be available in small hospitals. In our study, 50% of the involved hospitals would have been able to carry out the analysis autonomously,

directly or by making arrangements with a laboratory within the hospital. The extreme sensitivity of the PCR amplification technique can cause laboratory artifacts due to minimal carryover contaminations. This concern can be avoided by good laboratory practice, using dedicated consumables, performing the analysis within a hood, and including additional negative controls. It is also important to underline that identification of microbial markers by DNA amplification does not imply an infective capability due to the presence of alive pathogens, but can reveal the presence of contaminants from a previous patient, indicating a possible reprocessing failure. A further limitation is related to hypothetical drastic disruptions of the natural microbiota composition; for example, due to pathogens and/or antibiotic therapies that would have eradicated any of the bacteria species selected for the PCR analysis. This condition was not observed in any of the used endoscopes tested in our survey (confirmed by Metagenomics); nevertheless, it could be overcome by further increasing the number of detectable species. Lastly, NGS itself, even if it provided more informative output, would not be appropriate for routine monitoring, not only because it is highly expensive and rarely available in hospitals, but also because of the long time required for the analysis and bioinformatic interpretation of output. NGS can, however, represent an approach for confirming doubt results and studying particular cases of scientific or legal medical interest. Therefore, the proposed real-time PCR strategy appears to be the most suitable method for rapid and specific monitoring by DNA analysis. In the long-term, its application may also support the identification of critical points in local reprocessing procedures or their validation and implementation.

Conclusions

In conclusion, the results of our survey strongly support the application of real-time PCR-based assays in surveillance of reprocessing, especially in combination with the reported simplified protocol for mfDNA isolation using nylon-tipped flocked swabs+NAO® baskets. This promising approach deserves attention and further studies to confirm its reliability as a feasible support to flank traditional microbiology monitoring and update guidelines.

Acknowledgments

The authors thank Dr. Rachele Antonietta Mimmo (Presidio Ospedaliero “Antonio Cardarelli”, Campobasso, Italy); Dr. Sandra Savini (Azienda Ospedaliero Universitaria “Ospedali Riuniti”, Ancona, Italy), Dr. Anna Rita Mattaliano (Azienda Ospedaliera “Vittorio Emanuele”, Catania, Italy), and Dr. Luigi Cugia (Operative Unit of Gastrenterology, SS. Annunziata Hospital, Sassari, Italy) for supporting the survey. The authors also thank Dr. Elena Scaramucci for updating the references and editing the manuscript.

References

1. Aabakken L. Current endoscopic and pharmacological therapy of peptic ulcer bleeding. Best Pract Res Clin Gastroenterol 2008;22:243-59.
2. Kovaleva J, Peters FT, van der Mei HC, Degener JE. Transmission of infection by flexible gastrointestinal endoscopy and bronchoscopy. Clin Microbiol Rev 2013;26:231-54.
3. Rutala WA, Weber DJ, Healthcare Infection Control Practices Advisory Committee. 2008. Guideline for disinfection and sterilization in healthcare facilities. CDC, Atlanta, GA. Available from: http://www.cdc.gov/hicpac/pdf/guidelines/Disinfection_Nov_2008.pdf
4. Petersen BT, Chennat J, Cohen J, Cotton PB, Greenwald DA, Kowalski TE, et al. Multisociety guideline on reprocessing flexible GI endoscopes: 2011. Infect Control Hosp Epidemiol 2011;32:527-37.
5. Brandabur JJ, Leggett JE, Wang L, Bartles RL, Baxter L, Diaz GA, et al. Surveillance of guideline practices for duodenoscope and linear echoendoscope reprocessing in a large health system. Gastrointest Endosc 2016;84:392-9.
6. Nelson DB, Muscarella LF. Current issues in endoscope reprocessing and infection control during gastrointestinal endoscopy. World J Gastroenterol 2006;12:3953-64.
7. Ofstead CL, Wetzler HP, Doyle EM, Rocco CK, Visrodia KH, Baron TH, et al. Persistent contamination on colonoscopes and gastroscopes detected by biologic cultures and rapid indicators despite reprocessing performed in accordance with guidelines. Am J Infect Control 2015;43:794-801.
8. Alvarado CJ, Reichelderfer M. APIC guidelines for infection prevention and control in flexible endoscopy. Association for Professionals in Infection Control. Am J Infect Control 2000;28:138-55.
9. Rutala WA, Weber DJ. Reprocessing semicritical items: current issues and new technologies. Am J Infect Control 2016;44:53-62.
10. Nelson DB, Jarvis WR, Rutala WA, Foxx-Orenstein AE, Isenberg G, Dash GR, et al. Multi-society guideline for reprocessing flexible gastrointestinal endoscopes. Infect Control Hosp Epidemiol 2003;24:532-7.
11. Buss AJ, Been MH, Borgers RP, Stokroos I, Melchers WJ, Peters FT, et al. Endoscope disinfection and its pitfalls— requirement for retrograde surveillance cultures. Endoscopy 2008;40:327-32.
12. Associate of periOperative Registered Nurses. Guideline for processing flexible endoscopes. Denver (CO): Sterilization and Disinfection; 2016. p. 675-758.
13. Moses FM, Lee J. Surveillance cultures to monitor quality of gastrointestinal endoscope reprocessing. Am J Gastroenterol 2003;98:77-81.
14. Funk SE, Reaven NL. High-level endoscope disinfection processes in emerging economies: financial impact of manual process versus automated endoscope reprocessing. J Hosp Infect 2014;86:250-4.
15. Bringhurst J. Special problems associated with reprocessing instruments in outpatient care facilities. Am J Infect Control 2016;44:63-7.
16. Beilenhoff U, Neumann CS, Rey JF, Biering H, Blum R, Cimbro M, et al. ESGEESGENA guideline: cleaning and disinfection in gastrointestinal endoscopy. Endoscopy 2008;40:939-57.
17. Hanson PJ, Gor D, Clarke JR, Chadwick MV, Nicholson G, Shah N, et al. Contamination of endoscopes used in AIDS patients. Lancet 1989;2:86-8.
18. Sethi S, Huang RJ, Barakat MT, Banaei N, Friedland S, Banerjee S. Adenosine triphosphate bioluminescence for bacteriologic surveillance and reprocessing strategies for minimizing risk of infection transmission by duodenoscopes. Gastrointest Endosc 2017;85:1180-7.
19. Ofstead CL, Wetzler HP, Heymann OL, Johnson EA, Eiland JE, Shaw MJ. Longitudinal assessment of reprocessing effectiveness for colonoscopes and gastroscopes: Results of visual inspections, biochemical markers, and microbial cultures. Am J Infect Control 2017;45:26-33.
20. Valeriani F, Giampaoli S, Buggiotti L, Gianfranceschi G, Romano Spica V. Molecular enrichment for detection of S. aureus in recreational waters. Water Sci Technol 2012;66:2305-10.
21. Mansi A, Amori I, Marchesi I, Marcelloni AM, Proietto AR, Ferranti G, et al. *Legionella* spp. survival after different disinfection procedures: comparison between conventional culture, qPCR and EMA-qPCR. Microchem J 2014;112:65-9.
22. Valeriani F, Protano C, Gianfranceschi G, Cozza P, Campanella V, Liguori G, et al. Infection control in healthcare settings: perspectives for mfDNA analysis in monitoring sanitation procedures. BMC Infect Dis 2016;9:394.
23. Giampaoli S, Berti A, Valeriani F, Gianfranceschi G, Piccolella A, Buggiotti L, et al. Molecular identification of vaginal fluid by microbial signature. Forensic Sci Int Genet 2012;6:559-64.
24. Olympus USA. GI endoscope reprocessing. Available from: http://medical.olympusamerica.com/customer-resources/cleaning-disinfectionsterilization/cds-instructions/gi-endoscope-reprocessing. Accessed July 6, 2016.
25. Pentax Medical. Reprocessing step-by-step. Available from: https://www.pentaxmedical.com/pentax/en/95/1/Manual-Reprocessing/. Accessed July 6, 2016.
26. Centers for Disease Control and Prevention, CDC. Interim protocol for healthcare facilities regarding surveillance for bacterial contamination of duodenoscopes after reprocessing. Available from: https://www.cdc.gov/hai/pdfs/cre/interim-duodenoscope-surveillance-Protocol.pdf. Accessed April 3, 2015.
27. Kittelmann S, Seedorf H, Walters WA, Clemente JC, Knight R, Gordon JI, et al. Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. PLoS ONE 2013;8:e47879.
28. Giampaoli S, DeVittori E, Valeriani F, Berti A, Romano Spica V. Informativeness of NGS analysis for vaginal fluid identification. J Forensic Sci 2017;62:192-6.
29. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261-7.
30. Zhou Q, Su X, Ning K. Assessment of quality control approaches for metagenomic data analysis. Sci Rep 2014;4:6957.