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Dentistry — Test methods for dental unit waterline biofilm treatment

Médecine bucco-dentaire — Méthodes d'essais pour le traitement du biofilm dans les conduites d'eau de l'unit dentaire

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 106, *Dentistry*, Subcommittee SC 6, *Dental equipment*.

This first edition of ISO 16954:2015 cancels and replaces the first edition of ISO/TS 11080:2009, of which it constitutes a technical revision.

Dentistry — Test methods for dental unit waterline biofilm treatment

1 Scope

This International Standard provides type test methods for evaluating the effectiveness of treatment methods intended to prevent or inhibit the formation of biofilm or to remove biofilm present in dental unit procedural water delivery systems under laboratory conditions.

This International Standard does not apply to devices intended to deliver sterile procedural water or sterile solution. It also does not apply to lines, tubing, or hoses that deliver compressed air within the dental unit.

This International Standard does not establish specific upper limits for bacterial contamination or describe test methods to be used in clinical situations. It also does not establish test methods for evaluating any deleterious side effects potentially caused by treatment methods.

The test methods provided in this International Standard can be used to test other dental equipment that delivers non-sterile water to the oral cavity.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 1942, *Dentistry — Vocabulary*

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*

ISO 7494-1, *Dentistry — Dental units — Part 1: General requirements and test methods*

ISO 7494-2, *Dentistry — Dental units — Part 2: Water and air supply*

ISO 10523, *Water quality — Determination of pH*

ISO 19458, *Water quality — Sampling for microbiological analysis*

IEC 60601-1, *Medical electrical equipment — Part 1: General requirements for basic safety and essential performance*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in IEC 60601-1, ISO 1942, ISO 7494-1, and ISO 7494-2 and the following apply.

3.1
biofilm
structured community of microorganisms inhabiting a self-developed extracellular biopolymeric matrix attached to a surface

3.2
dental unit
combination of interconnected dental equipment and dental instruments constituting a functional assembly for use in the provision of dental treatment

[SOURCE: ISO 1942:2009, 2.86]

3.3
dental unit procedural water delivery system
system of components of a dental unit which convey water from a supply source to one or more outlets used for dental treatment

3.4
procedural water
water supplied by the dental unit for use in the oral cavity

EXAMPLE Handpiece procedural water, multifunctional syringe water, scaler procedural water, or rinse cup water.

[SOURCE: ISO 7494-2:2003, 3.1]

3.5
surrogate dental unit water system
test apparatus which accurately recreates the procedural water delivery system of a dental unit, including design, construction, configuration, and operation of all water-bearing elements of the procedural water delivery system, but not necessarily including other dental unit components which do not directly come in contact with or control the flow of procedural water

3.6
test water
water having specified chemical and physical characteristics used for testing prior to the addition of the specified bacterial challenge suspension

3.7
bacterial challenge suspension
consortium of specified bacteria suspended in a nutrient growth medium or buffered solution used to inoculate test water

3.8
inoculated test water
prepared aqueous suspension used in testing, containing specified amounts of sterilized test water and one or more bacterial challenge suspension(s)

3.9
test apparatus for the control group
apparatus used in testing in which no treatment method is applied and no antimicrobial material is present in the waterline components

3.10
test apparatus for the test group
apparatus used in testing in which a treatment specified by the dental unit manufacturer is applied and any antimicrobial materials specified by the manufacturer are present in the waterline components, unless otherwise specified in the test requirements

4 Treatment methods

Depending upon the specific technical approach of a treatment method and its intended benefits, the performance objectives of a dental unit procedural water delivery system treatment method can include one or both of the following:

— prevention or inhibition of biofilm formation on surfaces within the dental unit procedural water delivery system;

— removal of biofilm from surfaces within the dental unit procedural water delivery system.

This International Standard specifies separate test methods for each of the above performance objectives. These requirements can be expanded upon, for example to include additional replicates or test scenarios. Additions to the test method shall follow the general principles of this International Standard and be fully described in the test report.

5 Test water and bacterial challenge suspensions

5.1 Test water

This subclause specifies the preparation of test water prior to inoculation.

5.1.1 Reagents

5.1.1.1 Water, in accordance with ISO 3696:1987, grade 3.

5.1.1.2 Calcium chloride ($CaCl_2$), or an equivalent molar quantity of a calcium chloride hydrate.

5.1.1.3 Magnesium chloride ($MgCl_2$), or an equivalent molar quantity of a magnesium chloride hydrate.

5.1.1.4 Sodium bicarbonate ($NaHCO_3$).

5.1.1.5 Tryptic soy broth (TSB), 1/3-strength, 10,0 g tryptic soy medium per litre broth.

5.1.1.6 Sodium hydroxide (NaOH), 1 mol/l.

5.1.1.7 Hydrochloric acid (HCl), 1 mol/l.

5.1.2 Preparation of hardness stock solution 1

Dissolve 74,0 g of calcium chloride (5.1.1.2) and 31,7 g of magnesium chloride (5.1.1.3) in 1,00 l water (5.1.1.1). Hardness stock solution 1 shall be sterilized by heat or filter-sterilized using a 0,2 µm microfilter and used within 24 h or stored at (5 ± 3) °C for up to 6 months.

5.1.3 Preparation of hardness stock solution 2

Dissolve 56,0 g of sodium bicarbonate (5.1.1.4) in 1,00 l water (5.1.1.1). Hardness stock solution 2 shall be filter-sterilized using 0,2 µm microfilter and used within 24 h or stored at (5 ± 3) °C for up to 6 months. Hardness stock solution 2 is not to be heat sterilized.

5.1.4 Preparation of test water prior to inoculation

For each litre of test water to be prepared, add 1,00 ml of 1/3-strength TSB (5.1.1.5) and 1,80 ml of hardness stock solution 1 (5.1.2) to 1,00 l water (5.1.1.1) and steam sterilize. After the sterilized solution has cooled, for each litre of test water add 4,00 ml of hardness stock solution 2 which has been filter-

sterilized using a 0,2 µm microfilter. Adjust the pH to 7,0 to 8,0, measured according to ISO 10523, by adding sodium hydroxide (5.1.1.6) or hydrochloric acid (5.1.1.7). The test water shall be used within 24 h or stored at (5 ± 3) °C for up to one week.

NOTE 1 The hardness of the prepared test water is approximately 1,8 mmol of calcium ions per litre (equivalent to approximately 180 mg/l as $CaCO_3$). This corresponds to the upper limit of the generally accepted range for hard water.[17]

NOTE 2 The test water has a concentration of approximately 10 mg TSB per litre, which yields a total organic carbon (TOC) level of approximately 4 mg/l, although the exact TOC level might vary somewhat. This approximate TOC level is consistent with the recommended upper limit of 4 mg/l for TOC in chlorinated drinking water[1] and is included in the test water to reduce the time for biofilm formation.

5.2 Bacterial challenge

Bacterial challenge suspensions used to inoculate the test water shall be prepared with the following bacteria from the American Type Culture Collection (ATCC) or an international authorized ATCC distributor:

a) *Pseudomonas aeruginosa* (ATCC #700888);

b) *Klebsiella pneumoniae* (ATCC #13882).

Alternate strains of *P. aeruginosa* and *K. pneumoniae* (i.e. different ATCC numbers for the same bacterium species) can be substituted if the specified strains are not available, provided that the isolation source indicated by ATCC is water or a water system.

The bacterium species shall be separately reconstituted in sterilized dilute TSB, having a concentration of 0,3 g TSB per litre. The reconstituted cultures shall be used within eight transfer passages. Cultivation of the bacteria for inoculating test water shall be performed one day before preparing the inoculated test water in accordance with 5.3.

Compliance with appropriate laboratory safety practices is critical when working with these bacteria, including the handling of waste that can be contaminated with these bacteria.

5.3 Inoculated test water

On days of test apparatus operation, inoculated test water shall be prepared within two hours before the commencement of the daily flow program specified in 6.2.2 by inoculating sterilized test water (5.1) with both of the reconstituted bacterial cultures (5.2), to achieve a concentration of 5×10^1 CFU/ml to 5×10^2 CFU/ml of each of the bacteria and a total bacterial concentration of 10^2 CFU/ml to 10^3 CFU/ml in the inoculated test water. The temperature of the sterilized test water at the time of inoculation shall be (23 ± 3) °C.

To ensure accurate concentrations of each bacterium species in the inoculated test water, it can be useful to centrifuge and re-suspend each of the reconstituted bacterial cultures in sterile phosphate buffer and determine the approximate bacterial concentration using turbidimetric measurements. Using these results, the volume of each of the single-species bacterial suspensions to be added to the test water can be calculated. Alternatively, other methods for achieving the specified inoculation range can be used.

6 Test apparatus

The test apparatuses shall consist of a specified number of either dental units or surrogate dental unit water systems which closely replicate the dental unit procedural water system.

In order to achieve reproducible results, all components of the test apparatuses which are in contact with the procedural water shall be new each time the test procedure for biofilm prevention or inhibition

(7.2) or the sequence of test procedures for biofilm prevention or inhibition (7.2) and for biofilm removal (7.2) is performed.

NOTE As specified in 7.3.1, the test procedure for biofilm removal (7.3) is performed after first developing biofilm in the test apparatuses per the test procedure for biofilm prevention or inhibition (7.2).

6.1 Test apparatus design

6.1.1 General

If the test apparatuses consist of dental units, the dental units shall represent the most challenging model or configuration (when more than one model or configuration is available from the manufacturer). Length of waterlines, number of branch waterlines and likelihood of stagnation are among the factors that shall be considered in determining the most challenging model or configuration.

If the test apparatuses consist of surrogate dental unit water systems, the surrogate dental unit water systems must be able to simulate the basic clinical performance parameters of a functioning dental unit, including as described in 6.2. The surrogate dental unit water systems must represent the most challenging model or configuration of the dental unit which the surrogate dental unit water systems are intended to represent (when more than one model or configuration is available from the manufacturer). Surrogate dental unit water systems shall accurately recreate the procedural water delivery system, including design, construction, configuration and operation of the water-bearing elements of the procedural water delivery system. Other components which do not directly come in contact with or control the flow of procedural water need not be included, such as structural and decorative components. The components of surrogate dental unit water systems shall be subject to the same environmental conditions (i.e. light exposure and temperature) as components in the dental units which they represent.

Any air gap system or other backflow prevention device required to comply with the backflow prevention requirements of ISO 7494-2 for isolating the procedural water from the incoming water shall be included in the test apparatuses.

Critical elements to be recreated by the surrogate dental unit water systems includes the following:

— configuration of waterlines (placement of components, arrangement of branch lines, dead legs, etc.);

— tubing diameter(s);

— tubing length(s);

— tubing material(s);

— other components which contact procedural water or regulate flow (control blocks, valves, fittings, etc.);

— location of any water treatment devices (filters, automatic or passive treatment systems).

Dental units or surrogate dental unit water systems shall include at least one hose which in normal use supplies procedural water to a handpiece and one hose which in normal use supplies procedural water to a multifunctional (air/water) syringe. Hoses which normally supply procedural water to other instruments that are attached to the dental unit can be included in the dental units or surrogate dental unit water systems. Dental handpieces or other instruments or attachments that are normally removed from the dental unit and sterilized between patients shall be disconnected from the hose and not used in this test if practical.

EXAMPLE At point C according to ISO 14457:2012, Figure A.1.

Cuspidor bowl rinse waterlines shall be disconnected from dental units or excluded from surrogate dental unit water systems. Cuspidor cupfill waterlines can be included in dental units or surrogate dental unit water systems.

NOTE Cuspidor waterlines are permitted to be excluded based on the assumption that other waterlines will tend to be more challenging test environments for the removal, prevention, and inhibition of biofilm.

6.1.2 Considerations specific to antimicrobial materials and materials which prevent microbial adhesion

If the dental units or surrogate dental unit water systems to be tested include any antimicrobial materials or materials which prevent microbial adhesion in contact with the procedural water, the following modifications to the test apparatuses shall be implemented.

Test apparatuses for evaluating biofilm prevention or inhibition:

— Test apparatuses for the control group: Any antimicrobial materials or materials which prevent microbial adhesion in the dental units or surrogate dental unit water systems in the control group shall be replaced with materials that do not contain the antimicrobial agent(s) or adhesion preventive effect, but otherwise closely represent the antimicrobial material.

— Test apparatuses for the test group: The dental units or surrogate dental unit water systems in the test group shall include (if applicable) the antimicrobial material(s) or material(s) which prevent microbial adhesion.

Test apparatuses for evaluating biofilm removal:

— Test apparatuses for the test group: Any antimicrobial materials or materials which prevent microbial adhesion in the dental units or surrogate dental unit water systems in the test group shall be replaced with materials that do not contain the antimicrobial agent(s) or adhesion preventive effect, but otherwise closely represent the antimicrobial material. This is essential to evaluating the ability of the test treatment method to remove biofilm.

6.2 Test apparatus operation

6.2.1 Flow rates

The flow rate of the handpiece procedural water shall be adjusted to (30 ± 3) ml/min.

The water flow rate of the multifunctional (air)/water) syringe(s) shall be adjusted to (60 ± 6) ml/min.

If the test apparatuses include other instruments or devices supplied with procedural water, the flow rate(s) of those instruments or devices shall be adjusted according to manufacturer recommendations.

All flow rates shall be set prior to the start of the test. Flow rates shall be checked and adjusted if necessary at least once per week throughout the test period.

6.2.2 Flow patterns (on-off cycles)

The test apparatus waterlines shall be operated five consecutive days per week and set idle with no operation for two consecutive days per week. On days of waterline operation, inoculated test water shall be freshly prepared according to 5.3 and supplied to the test apparatuses. The water flow pattern shall be controlled by an automated daily flow program consisting of 30 cycles, which periodically operate the test apparatus waterlines according to the following schedule.

— The handpiece procedural water shall be operated for 30 sec per cycle. If more than one handpiece supplying procedural water is present, only one headpiece procedural waterline shall be operated during each cycle. The selected headpiece procedural waterline shall change sequentially with each cycle, assuring that each headpiece procedural waterline is operated approximately equally throughout the daily flow program.

— The syringe water shall be operated for 30 sec per cycle. If more than one syringe is present, only one syringe shall be operated during each cycle. The selected syringe shall change sequentially with each cycle, assuring that each syringe is operated approximately equally throughout the daily flow program.

— There shall be a period of 9 min per cycle without any water flow.

If instruments other than handpieces and syringes are included in the test apparatuses, their operation shall be incorporated into the daily flow program in a manner consistent with their intended clinical use.

6.2.3 Test environment temperature and preconditioning period

The temperature of the test environment shall be maintained at (23 ± 3) °C. All apparatuses shall be conditioned in this temperature range for at least 24 h before starting the test.

7 Test procedures

7.1 Testing sequence

Testing shall be performed in the following sequence:

a) Testing to evaluate biofilm prevention or inhibition according to 7.2;

b) Testing to evaluate biofilm removal according to 7.3.

NOTE This sequence reduces the quantity of test apparatuses and procedural steps by enabling the control group apparatuses having an established biofilm upon completion of 7.2 to be used as the test group apparatuses in 7.3.

7.2 Biofilm prevention or inhibition

7.2.1 General

Figure 1 depicts the test procedure for evaluating treatment methods that are intended to prevent or inhibit dental waterline biofilm formation.

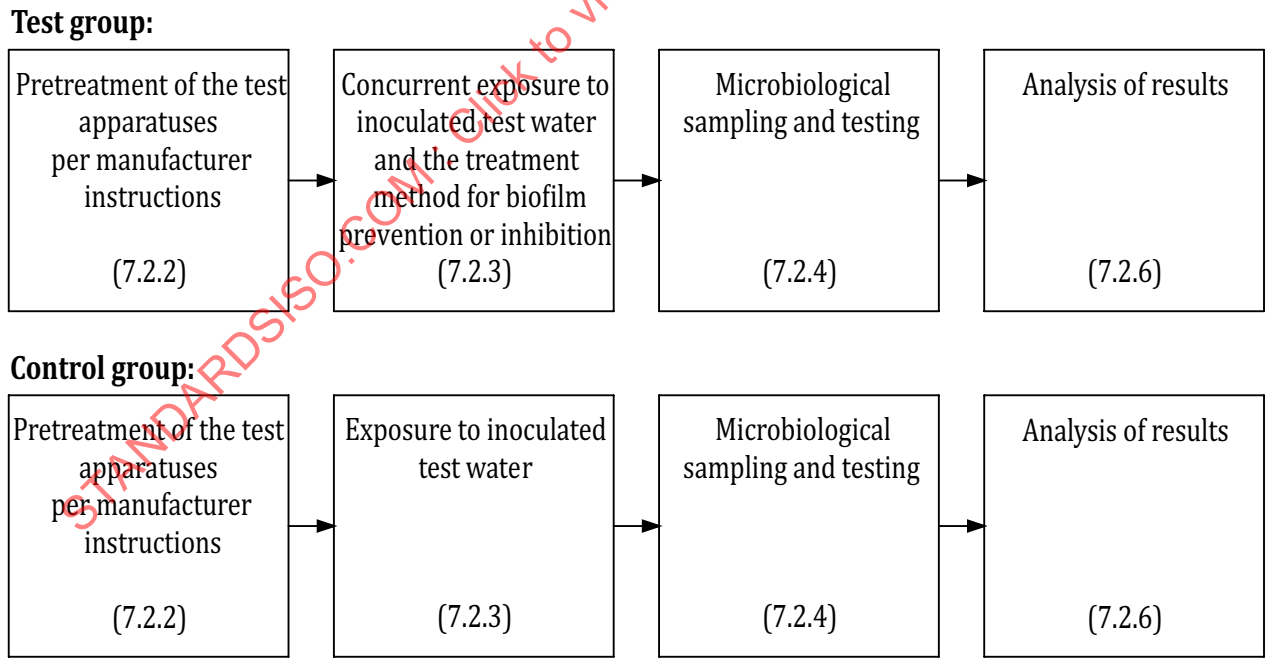

Figure 1 — Flow diagram for test method for evaluating biofilm prevention or inhibition

Since there is inherent variation in biofilm formation, testing shall be replicated on separate dental units or surrogate dental unit water systems. For the biofilm prevention and inhibition test method, at least two dental units or surrogate dental unit water systems shall be included in the test group and at least two dental units or surrogate dental unit water systems shall be included in the control group.

7.2.2 Pretreatment of the test apparatuses

All test apparatuses shall be pretreated according to the instructions of the manufacturer of the dental unit prior to testing if specified.

EXAMPLE If the instructions of the manufacturer indicate that a specified disinfection procedure is to be performed after the dental unit is installed and prior to use, the specified disinfection procedure shall be performed prior to performing this test method.

7.2.3 Concurrent exposure to inoculated test water and the treatment method for biofilm prevention or inhibition

For the test group, inoculated test water as specified in 5.3 shall be supplied to the dental units or surrogate dental unit water systems in conjunction with the treatment method per the manufacturer's instructions. For the control group, inoculated test water shall be supplied to the dental units or surrogate dental unit water systems without the treatment method. All dental units or surrogate dental unit water systems are to be operated according to 6.2. Inoculated test water shall be prepared daily within two hours of initiating the automated daily flow program. Microbiological sampling and testing of the inoculated test water supplied to the test apparatuses shall be performed in accordance with 8.1 before supplying to the test apparatuses at least once per week.

7.2.4 Microbiological sampling and testing

To determine the effectiveness of the treatment method, microbiological sampling and testing of all dental units or surrogate dental unit water systems in both the test group and the control group shall be performed in accordance with 8.1 and 8.2. Sampling and testing for total viable counts according to 8.1 shall be conducted on all dental units or surrogate dental unit water systems at least once per week throughout the duration of the test. Sterilized test water (without inoculum) shall be supplied to the test apparatuses when samples for microbiological testing are collected. A sufficient quantity of sterilized test water shall be allowed to flow to purge all waterlines of the test apparatuses. The sterilized test water shall then be allowed to set in the waterlines for 5 min prior to sampling for microbiological testing. Sampling waterline tubing and characterizing for biofilm formation according to 8.2 shall be conducted on all dental units or surrogate dental unit water systems at least once at the end of the test.

7.2.5 Test duration

Testing according to 7.2.3 and 7.2.4 shall continue until all of the following criteria have been met:

— at least four weeks of testing have occurred;

— a total viable count of at least 10^4 CFU/ml is measured in the effluent procedural water of all test apparatuses for the control group when sterilized test water is temporarily supplied to the test apparatuses instead of inoculated test water, flushing sterilized test water through the waterlines and waiting 5 mins before collecting the sample and then resuming normal operation with inoculated test water;

— the presence of biofilm is confirmed in all dental units or surrogate dental unit water systems in the control group according to 8.2 with all of the tubing samples in the control group classified as exhibiting either semiconfluent coverage or substantial, confluent coverage by microorganisms and/or biofilm.

After all of the above criteria have been met, the test procedure for evaluating biofilm removal according to 7.3 shall be performed as soon as practical. The daily procedure for exposure of the control group to inoculated test water specified in this subclause shall be continued until testing according to 7.3 is performed.

7.2.6 Analysis of results

The effectiveness of the treatment method to prevent or inhibit biofilm shall be characterized by reporting the following:

— log-transformed total viable counts in the effluent procedural water according to 8.1 (mean, standard deviation and number of replicates) in the test group and the control group at weekly intervals over the duration of the test;

— difference between the mean log-transformed total viable counts of the test group and the control group for each type of waterline outlet over the duration of the test;

— statistical analysis of log-transformed total viable count results comparing the test group to the control group using the two-tailed *t*-test and a significance criterion of $P < 0,05$ performed at the end of the test period;

— comparison of biofilm coverage for each type of waterline sampled in the test group and control group according to 8.2 performed at the end of the test period.

7.3 Biofilm removal

7.3.1 General

Figure 2 depicts the flow diagram for the test procedure for evaluating treatment methods that are intended to remove dental waterline biofilms.

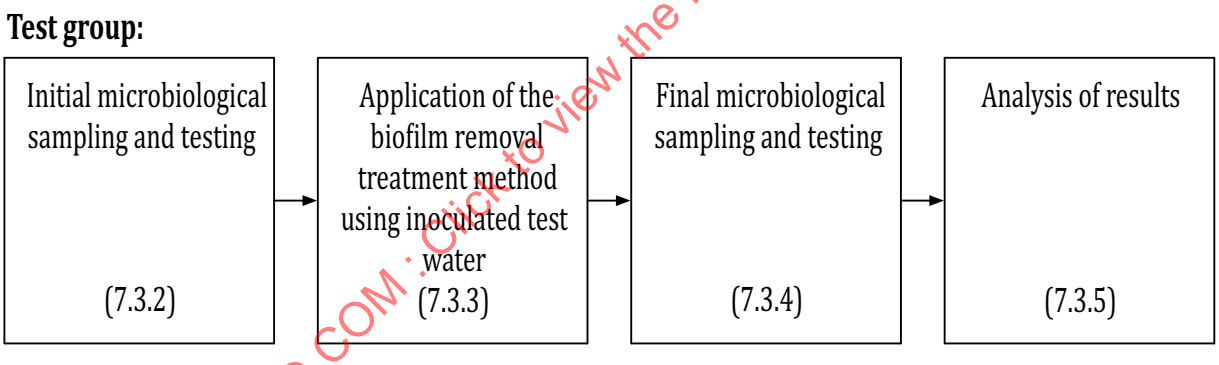

Figure 2 — Flow diagram for test method for evaluating biofilm removal

For the biofilm removal test method, at least two dental units or surrogate dental unit water systems shall be included in the test group and no dental units or surrogate dental unit water systems are required in a separate control group. The test group apparatuses shall consist of the control group apparatuses used in 7.2, which are specified to contain an established biofilm upon completion of 7.2.

NOTE No separate control group is required for the biofilm removal test method since the biofilm formation period prior to application of the treatment method serves the purpose of demonstrating that biofilm is capable of forming when no treatment method is applied.

7.3.2 Initial microbiological sampling and testing

Microbiological sampling and testing of all dental units or surrogate dental unit water systems shall be performed in accordance with 8.1 immediately prior to application of the biofilm removal treatment method. Sterilized test water (without inoculum) shall be supplied to the test apparatuses when samples for microbiological testing are collected. A sufficient quantity of sterilized test water shall be allowed to flow to purge all waterlines of the test apparatuses. The sterilized test water shall then be allowed to set in the waterlines for 5 min prior to sampling for microbiological testing.

Segments of waterline tubing from all dental units or surrogate dental unit water systems shall be sampled and characterized for biofilm in accordance with 8.2 immediately prior to application of the biofilm removal treatment method.

7.3.3 Application of the biofilm removal treatment method

The treatment method for removal of the established biofilm shall be administered in accordance with the instructions provided by the manufacturer of the dental unit or waterline treatment product.

If water is required for preparing or administering the treatment method, inoculated test water shall be used.

7.3.4 Final microbiological sampling and testing

To determine the effectiveness of the treatment method, microbiological sampling and testing of all dental units or surrogate dental unit water systems shall be performed in accordance with 8.1 and 8.2 after application of the biofilm removal treatment method. Sterilized test water (without inoculum) shall be supplied to the test apparatuses when samples are collected for total viable counts according to 8.1. A sufficient quantity of sterilized test water shall be allowed to flow to purge all waterlines of the test apparatuses. The sterilized test water shall then be allowed to set in the waterlines for 5 min prior to sampling for microbiological testing.

Segments of waterline tubing from all dental units or surrogate dental unit water systems shall be sampled and characterized for biofilm in accordance with 8.2 after application of the biofilm removal treatment method.

Consideration shall be given to specify the most appropriate time to sample per 8.1 and 8.2 after administering the treatment. If flushing with procedural water or other solution is specified by the manufacturer, this shall be performed before sampling and testing.

7.3.5 Analysis of results

The effectiveness of the treatment method to remove biofilm shall be characterized by reporting the following:

— log-transformed total viable counts in the effluent procedural water according to 8.1 (mean, standard deviation, and number of replicates) prior to and after administering the biofilm removal treatment method;

— reduction in log-transformed total viable counts in the effluent procedural water (i.e. numerical difference between the mean value prior to administering the treatment method and the mean value after administering the treatment method);

— statistical analysis of log-transformed total viable count results comparing the total viable counts before and after application of the treatment method using the two-tailed *t*-test and a significance criterion of $P < 0,05$;

— comparison of biofilm coverage for each waterline sampled prior to and after administering the biofilm removal treatment method according to 8.2.

8 Microbiological sampling and testing

8.1 Enumeration of bacteria levels in procedural water

8.1.1 Sampling

8.1.1.1 General

Sampling shall follow the guidance provided in ISO 19458. Enumeration shall begin within 24 h of sampling. Samples shall be stored at (4 ± 2) °C if enumeration cannot begin within 30 min of the sampling time.

All test samples shall be immediately treated at the time of collection to inactivate any residual antimicrobial agents, if applicable. Possible methods include administering a neutralizing agent or membrane filtering the test sample with a 0,2 µm rated membrane filter followed by rinsing with sterile phosphate buffer saline. If a neutralizing agent is used, its effectiveness and compatibility with the bacteria shall be confirmed with a neutralization study. Control group samples and test group samples shall be treated identically.

8.1.1.2 Sampling water supplied to test apparatuses

At specified sample times, one sample of at least 50 ml of the inoculated test water supplied to the test apparatuses shall be collected and analysed.

8.1.1.3 Sampling water output from test apparatuses

At specified sample times, one composite sample of procedural water shall be aseptically collected from the various water outlets on each of the specified dental units or surrogate dental unit water systems, with approximately equal volumes collected from each water outlet, such that the total volume of the composite sample is between 50 ml and 100 ml for each dental unit or surrogate dental unit water system. Alternatively, separate samples from each water outlet on each of the specified dental units or surrogate dental unit water systems can be collected and separately analysed.

8.1.2 Total viable count test procedure

Total viable counts shall be determined by the spread plate culture method.[12] Ten-fold serial dilutions from 10^0 (i.e. undiluted) to 10^{-3} (in sterilized phosphate buffer saline) of each test sample shall be prepared in triplicate. Fewer dilutions can be prepared in triplicate if the results are expected to fall in a certain range. 1,0 ml or 0,10 ml of each dilution shall be spread on R2A agar allowing adequate time for the sample to absorb into the agar before inverting and incubating aerobically at (23 ± 3) °C for seven days. After incubation, the colonies shall be enumerated and total viable counts shall be recorded as CFU/ml. The mean and standard deviation of the log-transformed total viable counts (i.e. base-10 logarithm of total viable counts) shall be reported for each set of samples.

8.1.3 Alternative total viable count test procedure

Planktonic bacteria levels can alternatively be enumerated by membrane filtration technique.[12] This method is appropriate, for example, if no practical method for inactivating residual antimicrobial agents in the samples is known or available. Three sets of 10-fold serial dilutions from 10^0 (i.e. undiluted) to at least 10^{-4} (in sterilized phosphate buffer saline) shall be prepared and 10 ml of each dilution shall be tested. Fewer dilutions can be prepared in triplicate if the results are expected to fall in a certain range. After membrane filtration of the sample or diluted sample, the membrane shall be rinsed twice by filtering 10 ml aliquots of sterilized phosphate buffer saline if antimicrobial agents can be present in any of the samples. The membranes shall be transferred to R2A agar, ensuring no air bubbles are trapped between the membrane and medium, and incubated aerobically at (23 ± 3) °C for seven days. After incubation the colonies shall be enumerated and recorded as CFU/ml. The mean and standard deviation of the log-transformed total viable counts shall be reported for each set of samples.

8.2 Biofilm on waterline surfaces

8.2.1 Sampling

At specified sample times, a segment of waterline tubing of at least 1 cm shall be sampled from near the outlet end of each waterline by cutting the tubing in an aseptic manner. The remaining lengths of tubing left on the test apparatus shall be reconnected using sterilized fittings to enable the test to resume, if appropriate.

The tubing sample shall be split longitudinally into approximately equal halves after fixation and before dehydration (see 8.2.2). To reduce the possibility of dislodging any biofilm from the tubing surface while splitting the samples longitudinally, the direction of cutting shall be parallel to the tubing axis.

8.2.2 Biofilm assessment test procedure

Scanning electron microscopy (SEM) shall be used to examine for biofilm coverage on the luminal surface of both halves of each tubing sample. Samples shall be prepared for SEM by fixing, dehydrating, drying, mounting, and sputter-coating with a conductive material in a manner suitable for SEM analysis of biofilm on tubing surfaces.

EXAMPLE The following represent one possible approach for the SEM preparation steps:

— Fixation of tubing samples in 2 % glutaraldehyde in 0,1 mol/l sodium cacodylate buffer at room temperature for a period of 30 min to 4 h, followed by three rinses with sodium cacodylate buffer without glutaraldehyde;

— Dehydration of tubing samples in a series of aqueous ethyl alcohol solutions of increasing ethyl alcohol concentration, including 30 %, 50 %, 70 %, 90 %, 95 %, and 100 %, for 10 min each;

— Critical point drying of tubing samples;

— Mounting of tubing samples to SEM stubs using double-sided conductive tape;

— Sputter-coating of tubing samples with gold-palladium.

The SEM instrument and choice of operating parameters shall be capable of producing images that resolve the specified bacteria used to inoculate the test water if present on the surface of the tubing samples.

Alternatively, environmental scanning electron microscopy (ESEM) can be used without fixation, dehydration, drying, or sputter coating prior to analysis.

Scan the tubing samples initially at a magnification of less than 1 000X. For each half of each tubing sample, select at least three locations which exhibit representative biofilm coverage (or lack thereof) for the sample and examine at a magnification between 1 000X and 5 000X. Capture and record representative photomicrographs at each of the selected locations.

For each tubing sample the biofilm coverage shall be qualitatively assessed according to the following categories:

— no microorganisms and no biofilm;

— isolated occurrence of microorganisms and/or biofilm (i.e. typically less than approximately 10% coverage over the entire surface of the tubing specimen);

— semiconfluent coverage by microorganisms and/or biofilm (i.e. typically less than approximately 50% coverage over the entire surface of the tubing specimen);

— substantial, confluent coverage by microorganisms and/or biofilm (i.e. typically greater than approximately 50% coverage over the entire surface of the tubing specimen).

A representative image of the most heavily covered region of each tubing sample shall be included in the test report.

