Haemoglobin as analyte for evaluation of cleaning

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Medical device cleaning should be conducted with validated processes (1) that necessitate objective assessment of the removal of unwanted substances from their external and internal surfaces. The analyte used for objective assessment of cleaning is protein. Since surgical instruments are generally contaminated with blood, haemoglobin can also be an important and revealing analyte for assessment of cleaning. In this investigation, the colour change of microhaematuria test strips was put in relation to the amount of haemoglobin contained in test solutions. The test strips proved to be very sensitive, and according to the evaluation of cleaning after elution of the instrument surfaces with 1% SDS solution, a negative haemoglobin test generally means that protein has been sufficiently removed from the instrument.
For instruments harbouring blood contamination, the use of test strips to assay SDS eluates for microhaematuria is a sensitive, easy to use, rapid test for investigation of the cleaning efficacy.

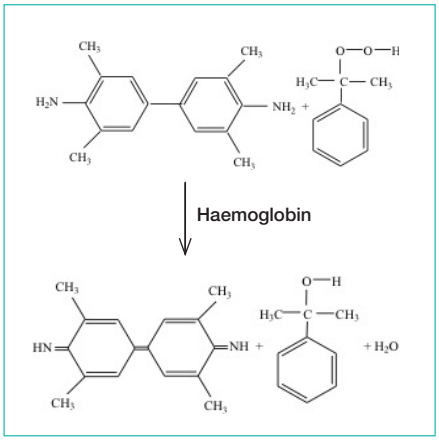

Fig.1: Haemoglobin pseudoperoxidase reaction converts tetramethylbenzidine to a coloured chromophore

Introduction

Medical device cleaning should be conducted with validated processes (1) that necessitate objective assessment of the removal of unwanted substances from their external and internal surfaces to the extent required for their reuse. Since it is well known that, on the one hand, inadequately cleaned medical devices may not be fully amenable to disinfection or sterilization and, on the other hand, the degree to which interfering substances are to be removed remains unknown, the guiding principle is to use optimized practices in accordance with the state of the art (2). The cleaner the medical devices, the more effective the disinfection and sterilization results and the greater their suitability for reuse.
The analyte used for objective assessment of cleaning is protein since this represents the contaminant substance group most commonly encountered when performing surgical procedures for patients. As such, it is featured in the pertinent recommendations, guidelines and standards (3, 4). Since surgical instruments are generally contaminated with blood, haemoglobin can also be an important and revealing analyte for assessment of cleaning. Thanks to the availability of analytical rapid tests, haemoglobin assays can also be used for quick routine testing in the everyday setting.
There are a number of standard methods for determination of the haemoglobin content of blood. Since the haemoglobin in blood is present in the form of different haemoglobin variants, photometric absorption measurement alone at a particular wavelength, e.g. 550 nm, is not sufficiently precise. Therefore the haemoglobin variants must first be converted to a suitable form, e.g. to cyanmethoglobin or to an alkaline hematin detergent (AHD) complex (5). These methods have limits of determination in the region of a few hundred µg haemoglobin per millilitre and are not sufficiently sensitive for assessment of cleaning. Therefore for evaluation of cleaning after elution of the instrument surfaces with 1% sodium dodecyl sulphate (SDS) solution, commercial test strips used to test for microhaematuria were employed (6, 7). Together with a tetramethylbenzidine (TMB) test these test strips are described in Annex J of ISO/TS 15883-5 as being adequately sensitive semi-quantitative methods (8). These methods embody a chemical detection reaction to the haemoglobin present in intact as well as in haemolysed red blood cells (RBCs) based on the pseudoperoxidase activity of haemoglobin. This reaction is illustrated in Figure 1. In the presence of a peroxide, such as cumolhydroperoxide or dimethylhexandihydroperoxide, chromogenic tetramethylbenzidine is oxidized to a blue-green chromophore if haemoglobin is present. Here, haemoglobin acts like an enzyme (peroxidase) in that it cataly-

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ses the oxidation reaction without being an enzyme itself. Hence this property of haemoglobin is also termed pseudoperoxidase activity.

This pseudoperoxidase test can only be used if the instruments had actually been contaminated with blood. If oxidative cleaning processes had been used the test cannot, or only under certain conditions, be used since even minute trace amounts of oxidizing agents could, depending on the pH value, result in a colour change, thus incorrectly indicating the presence of haemoglobin and producing false positive results. Besides, the pseudoperoxidase activity is also affected by conditions of the reprocessing process. Heat treatment and high alkalinity lead to some chemical changes in haemoglobin and to declining activity (9).

In the course of a field test where 510 Crile clamps, used as process challenge devices (PCDs), were soiled with blood in a standardized manner and their cleaning results evaluated after sample recovery by thoroughly rinsing with 2 ml 1% SDS solution in accordance with the biuret/BCA method as well as with microhaematuria test strips, it was demonstrated with there was a strong correlation and that the test strips were suitable for orientational assessment purposes (10, 11).

In the literature there are a number of reports of assays involving quantitative haemoglobin determination. These reports either specify only the analyzer system protocol without giving any details of the determination method or only the TMB method is mentioned, again without any further details (12, 13). There is even a recommendation that reference be made to a specific quantitative surface area as an acceptance criterion for cleaning; this was set at 2.19 µg haemoglobin per cm² (14).

This paper describes a simple method of haemoglobin quantification in solutions, such as those obtained through sample recovery based on SDS elution, aimed at demonstrating the relationship (i.e. correlation) between the colour change in the haemoglobin field of the test strips and the haemoglobin content of the eluate with respect to a specific surface area and the corresponding acceptance value, now a matter of topical debate.

Material and Methods

As quantitative colorimetric method of haemoglobin determination, a method based on oxidation of 3,3‘,5,5‘ tetramethylbenzidine was likewise used (15). That easy to use assay uses the Turbo TMB substrate (Pierce No. 34022). This is ready to use and contains TMB and a stabilized peroxide which must be stored at 4 °C and brought to room temperature before use.

The haemoglobin determination assay is very easy to conduct. Aliquots of 800 µl Turbo TMB were transferred to 200 µl of a sample containing haemoglobin or hematin and left to react for exactly 30 minutes at room temperature. Depending on their haemoglobin content, samples containing haemoglobin will give rise to a blue colour change. To terminate the catalytic reaction, after the 30 minutes have elapsed 1000 µl 2M sulphuric acid (VWR No. 198154D) was pipetted to the sample and mixed. The reaction with the acid gives rise to a yellow compound with maximum absorption at 450 nm. At that wavelength the photometric absorption values of the solutions were measured after three minutes and compared to that of pure water. The zero sample result, i.e. the reaction mixture with 200 µl 1% SDS, as the mean value of three measurements, was subtracted from the sample measurement results.

The calibrator used was pure hemin chloride (Sigma, H9039-1G, with 50 mg aliquots dissolved in 10 ml of a solution of 0.02 N sodium hydroxide/1% SDS. The solution was first diluted 1000-fold and this as well as 1:1 dilutions of the same used for calibration. 1% SDS solution, pH 11, was used for dilution.

To calculate the haemoglobin content it must be borne in mind that hemin chloride as a monomer has a molecular weight of 651.94 g and haemoglobin as a tetramer a molecular weight of 64460 g. The hemin contents must therefore be multiplied by the factor 24.72 to obtain the haemoglobin content.

The dilution stages with the lowest hemin content were assayed with the microhaematuria test strips (Medi-Test Combi-5, Macherey – Nagel), with only the terminal reaction field for haemoglobin being of relevance here. After immersion in the solution for 30 seconds the pseudoperoxidase reaction leads to a change in colour from yellow to yellow-green in the presence of around 10 RBCs/µl, to blue-green for around 50 RBCs/µl and dark blue-green for around 250 RBCs/µl (Fig.2)

Results

The 5 µg/ml hematin chloride solution was diluted in five stages 1:1 with 1% SDS solution to 0.078 µg/ml. That corresponded to haemoglobin concentrations of 123.75 – 61.88 – 30.94 – 15.49 – 7.75 – 3.88 µg/ml.The highest concentration based on the TURBO TMB method produced after the addition of sulphuric acid a deep yellow solution, which became brighter with lower concentrations, and at 15.49 µg/ml

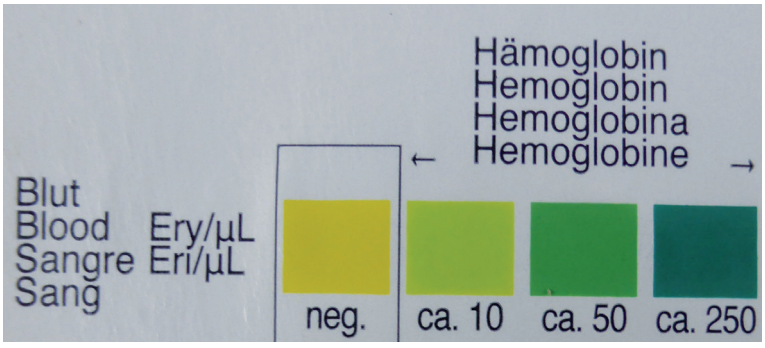

Fig. 2: Colour comparison chart for the haemoglobin reaction zone in the Medi-Test Combi-5

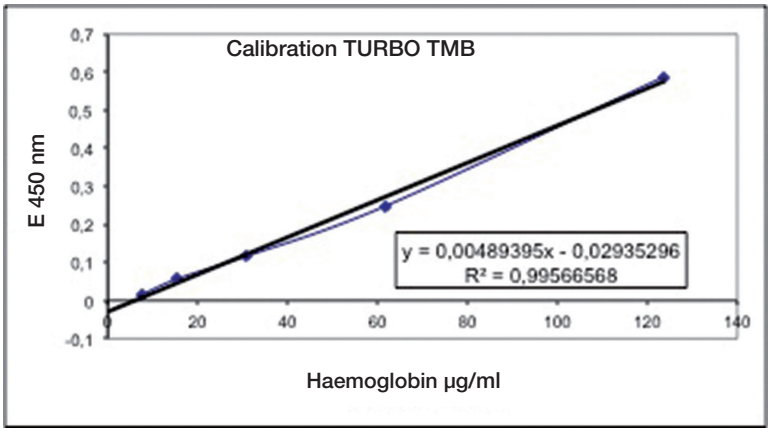

Fig.3: Calibration of the Turbo TMB method with hemin chloride which is converted to the equivalent haemoglobin concentration.

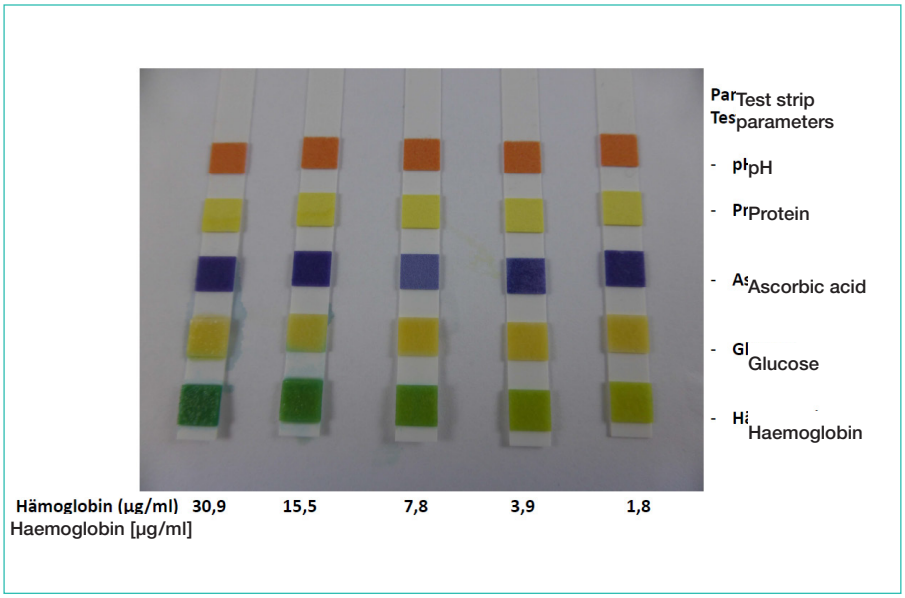

Fig.4: Colour changes in the haemoglobin reaction zone in relation to the haemoglobin concentration

produced only a pale yellowish solution. Already this serves as a guide for optical assessment of the haemoglobin content. The zero sample had an extinction of 0.273 +/– 0.006 and the sample with 3.88 µg/ml as haemoglobin an extinction of 0.271 +/– 0.011. The range beneath 10 µg haemoglobin/ml was associated with major fluctuations in the extinction measurements, probably due to concentration effects related to pipetting tolerances. Measurements for the yellow solutions were consistent and it appears prudent to use only those reaction mixtures with at least a bright yellow colour change for photometric evaluation. The intrinsic extinction measurements were negligible for the sample concentrations (<0.004) (Fig.3).

The hematin chloride solutions with the equivalent haemoglobin concentrations shown above were tested for microhaematuria using the test strips, while immersing only the terminal haemoglobin field in in the solution. The other parameters of the test strips are not relevant and besides the reaction zone for protein is not sensitive enough for analysing SDS eluates. The test strip haemoglobin zone proved to be very sensitive, and already the 3.9 µg/ml haemoglobin concentration produced a bright green colour change, corresponding to 10 RBCs per µl. In the 7.75 µg/ml to 15.5 µg/ml haemoglobin range, a blue-green colour was formed corresponding to 50 RBCs per µl (Fig. 4).

Discussion

The TURBO TMB method as applied here has a limit of determination of around 8 to 10 µg per ml eluate. That is sensitive enough to detect residual amounts greater than 1.0 µg per cm² on, for example, a Crile clamp that had been eluted in accordance with the DGKH, DGSV and AKI Guideline (18) with 2 ml 1% SDS solution, while investigating a surface area of just less than 20 cm². The microhaematuria test strips proved to be much more sensitive. The package insert supplied with the rapid test states that a change in colour can first be detected at 0.15 µg per ml. But when using such test strips in combination with a reflectometry method it was possible to quantify even haemoglobin contents in this nanogram range (16). The test strips are thus a very sensitive, easy to use and inexpensive means of testing for the presence of haemoglobin in SDS eluates when cleaning medical devices that had been contaminated with blood. If no colour change is seen in the reaction zone, there is no point in quantification. Only those eluates for which the reaction zone shows a pronounced blue-green colour change should be subjected to quantitative evaluation of the haemoglobin content. The Turbo TMB method, inter alia, can then be used for quantification.

As stated above, among the methods used in a field test aimed at formulation of acceptance criteria for the tolerable amounts of residual protein on Crile clamp PCDs as per the DGKH, DGSV and AKI Guideline, was the assay based on microhaematuria test strips (10, 11). Of the 510 instruments investigated, for residual protein amounts of over 100 µg, in 92% of cases the test strips produced a dark blue-green change in colour reflecting a haemoglobin content of over 30 µg per ml eluate. For instruments harbouring less than 50 µg residual protein, no colour change was seen in the haemoglobin reaction zone of the test strips in 96.6% of cases (Fig. 5).

The Crile clamps had a total surface area of around 42–44 cm² (17). For elution as per the DGKH, DGSV and AKI Guideline

Results of the haemoglobin test strips in correlation to the amount of residual protein

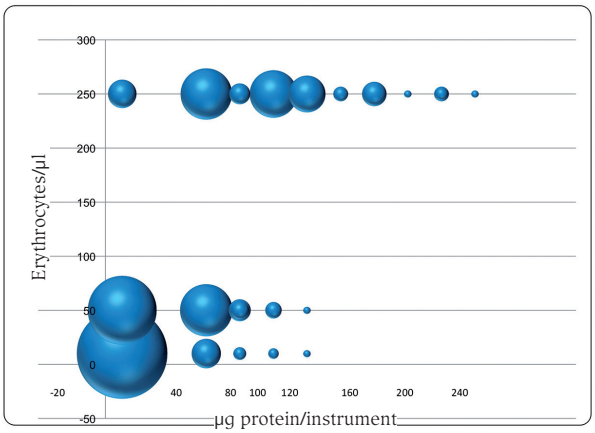

Fig. 5: Frequency of haemoglobin and protein findings

around half the surface area was investigated (only the functional area, including the joint) (18). That area was just below 20 cm². With reference to the recommendation to use as acceptance criterion 2.19 µg haemoglobin per cm² for assessment of the cleaning efficacy, 43.8 µg haemoglobin would then be acceptable in the eluate of 2 ml 1% SDS solution used for sample recovery from the clamps. That thus amounts to 21.9 µg per ml eluate, which in the test strips would produce an intense blue-green change in colour. That colour change when referred to the field test corresponds more to residual protein amounts of markedly more than 60 µg, which, given an acceptance criterion of <3 µg protein per cm², would no longer be acceptable. Here the Guideline specifies 50 µg as an acceptance value. Based on these results obtained for the Crile clamps and the experience gathered from routine use of the test strips, only a bright green colour change for eluates from properly cleaned medical devices would be acceptable. This is <7.8 µg haemoglobin per ml or at least <10 µg per ml. As such, at best only around 1 µg haemoglobin per cm² or less could be used as acceptance criterion. The data on which the 2.19 µg/cm² proposal was based were collected during a study of 30 flexible endoscopes (12). But that very limited sample size cannot be interpreted as being representative of all medical devices, and certainly cannot be applied to surgical instruments made of stainless steel, such as arterial clamps. Besides, the proposed acceptance criterion would not be much lower than that used for the tolerable residual protein amount. That cannot be right since we know from experience that after cleaning blood contamination, protein residues such as fibrin films still persist and that the haemoglobin was very effectively extracted.

For instruments harbouring blood contamination, the use of test strips to assay SDS eluates for microhaematuria is a sensitive, easy to use, rapid test for investigation of the cleaning efficacy. This should therefore be viewed as an important method for routine investigation of the cleaning efficacy for blood contaminated instruments in everyday practice. The 1% SDS solution and test strips needed can be sourced from the hospital pharmacies. An important point to bear in mind is that when using thermal processes the test instruments must be withdrawn from the washer-disinfector before the disinfection stage. Unfortunately, this interruption of the reprocessing process for routine control purposes is generally seen as a problem where process conduct and documentation are concerned and needs to be resolved.

References

1. Verordnung über das Errichten, Betreiben und Anwenden von Medizinprodukten (MPBetreibV) BGBL.I
2. Michels W: Acceptance criteria for the cleaning of medical devices. Forum 2015; Vol. 25: 9–10.
3. Anforderungen an die Hygiene bei der Aufbereitung von Medizinprodukten (KRINKO/BfArM). Bundesgesundheitsblatt 2012; 55: 1244–1310.
4. EN ISO 15883-1: 2006/Amd1: 2014 – Washer-disinfectors – Part 1: General requirements, terms and definitions and tests.
5. DIN 58931: 2010-8 – Hämatologie – Bestimmung der Hämoglobinkonzentration im Blut.
6. Michels W: Evaluation of a quick test for examining cleaning efficiency of processed surgical and minimally invasive instruments. Hyg Med 1997; 22 (4): 173–184.
7. Fengler TW, Pahlke H, Bisson S, Michels W: Are processed surgical instruments free of protein? Zentr Steril 2001; 9 (1): 20–32.
8. ISO/TS 15883-5: 2005 – Washer-disinfectors – Part 5: Test soils and methods for demonstrating cleaning efficacy.
9. Michels W: Detektionsmethoden der EN ISO 15883 für die Reinigungsprüfung in der Praxis. Aseptica 2006; 12 (2): 7–9.
10. Roth K, Michels W: Inter-hospital trials to determine minimal cleaning performance according to the guideline by DGKH, DGSV and AKI. Zentr Steril 2005; 13 (2): 106–116.
11. Michels W: Leistungsprüfung der Reinigung – Probleme der Praxis und Alternativen. Aseptica 2010; 16 (4): 14–16.
12. Alfa MJ: Worst-case soiling levels for patient-used flexible endoscopes before and after cleaning. Am J Infect Control 1999; 27: 392–401.
13. Alfa MJ, Olson N, Al-Fadhaly A: Cleaning efficacy of medical device washers in North America healthcare facilities. J Hosp Infect 2010; 74: 168–177.
14. A compendium of processes, materials, test methods and acceptance criteria for cleaning reusable medical devices. AAMI TIR30: 2011.
15. Huy NT et al.: An improved colorimetric method for quantitation of heme using tetramethylbenzidine as substrate. Anal Biochem 2005; 344: 289–291.
16. Frey J et al.: Hemoglobin assay for validation and quality control of medical device reprocessing. Anal Bioanal Chem 2015; 407: 6885–6889.
17. Michels W, Roth K, Eibl R: Assessment of cleaning efficacy based on the protein-surface relationship. Zentr Steril 2013; 21: 212–215.
18. Leitlinie von DGKH, DGSV und AKI für die Validierung und Routineüberwachung maschineller Reinigungs- und thermischer Desinfektionsprozesse für Medizinprodukte (4. Auflage). Zentr Steril 2014 Supplement