Evaluation of antiseptic disinfectant activity with static light scattering technology

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Summary

Background and aims. Healthcare-associated infections (HAI) are an increasingly important issue, for this reason disinfection and antisepsis practices acquire importance. The use of products with antiseptic activity and reports of resistance to these molecules, pose the need to test, in the local area, the sensitivity pattern. The aim of this study is to verify the ability of the analytical system Alfred 60AST (Alifax Spa Isola dell'Abbà, Polverara - PD - Italy) in evaluating the antimicrobial effectiveness *in vitro* of different molecules in comparison to the membrane filtration reference method, in accordance with the procedure NF T72- 152 proposed by the *Association Française de Normalisation* (AFNOR).

Materials and Methods. We used four antiseptic-disinfectant substances, commonly used in hospital practic: Iodopovidone, Ethanol, Chlorhexidine and DECS. ATCC strains were assessed both with clinical isolates. The eventual development occurs by means of microbial ALFRED AST60 was carried out in progressive times (30, 60 and 120 minutes) with different dilutions for each of the disinfectant molecules tested.

Results and Conclusions. Comparison tests carried out between membrane filtration method and instrumentation Alfred 60AST gave results almost totally concordant. The analyzer Alfred 60AST can then be appropriately adapted to the *in vitro* evaluation of antiseptics, representing a valuable aid in the periodic monitoring of their activities and the prior assessment of sensitivity for therapeutic use. Though preliminary, the study confirms the existence of bacteria resistant to alcohols and biguanides, and it emphasizes the opportunity to verify the *in vitro* sensitivity profile.

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Introduction

Healthcare-associated infections (HAI) constitute an increasing relevance problem with serious consequences for the patient; worrisome ecological alterations, complexity of clinical and care management and also economic repercussions. For this reason, the practices related to disinfection and antisepsis acquire rising importance for an integrated management that allows the best environmental sanitation and the appropriate use of such antimicrobial molecules. Disinfection and antisepsis procedures eliminate or inactivate microorganisms respectively from contaminated inert substrates or organic surfaces; ensuring bactericidal activity after short contact times at room temperature, thanks to an unspecific physical-chemical mechanism (9). Nowadays the use of antiseptic activity products is recommended in accordance with Multi Drug Resistant microorganisms (MDR) colonization care protocols. Furthermore, the arising resistances pose the compelling need to verify, at a local level, the sensitivity patterns to commonly-used antiseptic and disinfectant molecules. The objective of this assessment is to verify the analytical system Alfred 60AST (Alifax Spa Isola dell'Abbà, Polverara - PD - Italy) ability to evaluate *in vitro* antimicrobial efficacy of different disinfectant and antiseptic molecules, in comparison to the membrane filtration reference method.

Materials and Methods

Antimicrobial agents

We used four antiseptic substances commonly implied in hospital practice, classified into four distinct chemical groups: halogenated, oxidizers, biguanide added with alcohols and alcohols.

Halogenated

We used the halogenated derivative Iodopovidone (IP), with antiseptic bactericidal and sporicidal activity, into commercial formulation *ESO JOD 10% cutaneous solution* (Esoform Manufacturing Srl, Rovigo). The active ingredient is Iodopovidone (1% active iodine in 100 g) (3). Elementary iodine

is combined with the povidone, a chemically inert polymer which acts as a solubilizer. It is able to perform its extended spectrum antimicrobial activity through alterations in the synthesis of microbial proteins, modifications of the lipids chemical-physical properties and immobilization of the membrane.

Oxidising

We used the derivative of active chlorine *DECS Ambiente (DECS) 2.7% cloro attivo* (Lombarda H Srl, Albairate - Milano). It is a directly oxidant disinfectant, clear, straw-colored, alkaline (pH 12±0.5). 100 mL of solution containing 2.8 g of sodium hypochlorite, as active ingredient. The electrolytic chlorine is able to inhibit the sulfhydryl-enzymatic systems, which are essential for the microbial metabolism, by thiol groups irreversible oxidation (6;8).

Biguanides

We used the commercial formulation Neoxinal Alcoholic 0.5% +70% cutaneous solution (New Farmec Srl, Settimo di Pescantina - VR) (5) labeled CLX. The active ingredient is chlorhexidine gluconate (0.50 g to 100 g of solution) to which is also added ethyl alcohol 96%. The positive charge molecular structure, with lipophilic groups, causes damage to the cytoplasmic microorganisms membrane, that undergo loss of low molecular weight components, massive coagulation of the cytoplasm and precipitation of cellular proteins and nucleic acids.

Alcohols

Ethanol 96° (RPE Carlo Erba Reagents S.r.l., Cornaredo - MI) exerts its biocide activity, as all alcohols, through a protein denaturation mechanism that occurs, however, in the presence of a certain percentage of water. By convention is, hereinafter, defined EtOH.

Bacterial strains

UOC Microbiology and Virology, ASST Papa Giovanni XXIII, Bergamo, has verified the microbicide capacity of four solutions against four ATCC microbial strains: *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. These strains were tested with the reference method (membrane filtration) and also the analyzer Alfred 60AST (Alifax Spa, Italy).

Furthermore, 20 microbial strains, from the UOC Microbiology and Virology collection, were also exclusively tested with the analyzer Alfred 60AST: *E. coli* ESβL (4) CPE-producing *Klebsiella pneumoniae* (3), *P. aeruginosa* MDR (3), *Acinetobacter baumannii* MDR (3), Vancomycin-resistant *Enterococcus faecium* (3), Methicillin-resistant *S. aureus* (4).

Reference tests

Minimum Bactericidal Concentration (MBC) was determined for each microbial strains using filtration membrane method, to determinate antiseptics and disinfectants bactericidal activity, in accordance with NF T72-152, procedure proposed by the *Association Française de Normalisation* (AFNOR) (1). Experimental tests involved the execution of a preliminary test, aimed at checking the technical feasibility. Following, we set up a proper test, intended to verify the antiseptic-disinfectant effectiveness of the products.

Test with static light scattering technique

ALFRED 60AST (Alifax SpA, Italy) is an automated system for bacterial culture and for antibiotic sensitivity tests. This appliance uses light scattering technology, detecting rapidly the presence of bacteria and possible resistances, with declared sensitivity and specificity equal to 96.64% and 88.25%, respectively. The sensitivity is directly proportional to the time of analysis: for a 6-hour test it reaches ≤50 CFU/mL values (7). The instrument monitors the growth phase of the basic inoculation bacteria, performed in appropriate broths. The level of broth turbidity is detected by the McFarland Monitor: when the sample reaches 0.5 McF value, it is placed in a refrigerated area of the instrument and tested against a default panel of antiseptic-disinfectant substances. The technique of Static Light Scattering measures the scattered light average intensity over time (10-30 seconds) as a function of the sample concentration. The average time removes scattering signal fluctuations. The intensity of the light diffused by a macromolecule is proportional to the product between the average molecular mass and its concentration, defined by the intensity/mass ratio. The ALFRED 60AST instrumentation returns in real time, as a result of growth broths monitoring, a graph with two curves: actually it monitors the scattering by 2 detectors, placed at two different angles, 90° and 30°. The first angle determinates all particles suspended in the sample, thus also any signal disturbing components (*i.e.*: pigments). Whereas, the acute angle exclusively detects bacterial cells, thus enabling an effective discrimination of actual growth that is taking place (or not). The graph shows on the abscissa the time elapsed (expressed in hours and minutes), while on the ordinate are shown the CFU/mL: the curves link sample microbial growth and incubation time spent. ALFRED 60AST methodology involves the preparation of a subculture at 0.5 McF, as described below: a colony of each strains (reference and clinical isolates) was inoculated into two test tubes, each containing 2 mL of eugonic broth. Inoculated broths were then incubated in ALFRED 60AST instrumentation. From bacterial suspensions broths with a concentration equal to 0.5 McF, defined *witness*, were carried out broth serial dilutions up to values 10, 10^2, 10^3 CFU/mL, verified with reseeding on Blood Agar. The second test tube inoculated with the colony was left in incubation in the instrument for the whole duration of the test, in order to monitor the growth of the microorganism in a lacking inhibitory substances medium: for this reason it is defined *free growth witness* or *reference*. Subsequently, tubes containing 2 mL of eugonic broth, for each disinfectant and for each concentration tested, were inoculated with 100 µL of a *free growth witness*. Each test was performed in duplicate. The four antimicrobial substances were tested at different concentrations of the commercial product: ethanol at 50, 25 and 12% concentrations, CLX at 2.5, 2 to 1.25%, the DECS 5, 2.5 to 1.25% and, finally, IP 25, 12.5, 5 and 2.5%. The verify of the possible microbial development, in progressive time (30, 60 and 120 minutes), was carried out by plating 1 µL of each tube with recorded signs of growth. Moreover, 100 µL of all the broths were placed in culture on Blood Agar media. CFU count was calculated after 24 hours of incubation, in order to confirm the CFU/mL value returned by the instrument. For wild type strains testing, we selected two different dilutions (500 and 1000 µL in 2 mL of broth) for each disinfectant molecules. The second dilution chosen for IP was 400 µL and not 500 µL in 2 mL of broth, to solve the disturbing phenomena of scattering.

Results

Reference method results

For each individual tests performed, it was executed a check defined "free growth witness", obtained in the same experimental conditions, without the addition of the antibacterial substance. All four antibacterial molecules presented bactericidal effect on all ATCC strains (*E. coli*, *S. aureus*, *P. aeruginosa* and *E. faecalis*) used at a concentration of 50%. Overall results are carried out in Table 1.

Light scattering method results

Free growth witnesses and dilutions CFU count confirmed 10^8 CFU/mL for each of the ATCC strains tested. Figure 1 shows, by way of example, the diagram for *S. aureus* ATCC 29213 obtained with the instrument ALFRED AST60. The appropriate dilution to be used for the wild strains analysis follows the comparison of the ATCC strains behavior in the presence of different amounts of each antiseptic-disinfectant substances. The results tabulated (Table 2) refer to bacterial growth or absence on Agar Blood. *Killing effect* is defined as the decrease in the number of bacteria (measured as CFU/mL) as a function of time, while with *bacteriostatic effect* is meant the inhibition of bacterial growth, therefore the inoculated bacteria number is constant confirmation.

It is emphasized that Iodopovidone dilution of 500 µL (25% concentration) exhibits a strong disturbance of scattering during initial readings, which completely disappears over time because of the disinfectant lap dissolve in the culture broth, retrieving back the original pale yellow color which does not *disturb* the instrument (Figure 2).

Clinical MDR (Multi Drug Resistant) isolates strains results

For MDR strains are selected two different dilutions for each disinfectants. The first dilution is the lowest at which it is observed total lack of growth, the second is the previous to such dilution, *i.e.* the more concentrated. Table 3 reports test results for each species taken into consideration.

The growth or lack of growth in the plate corresponded to the results obtained with the instrumentation ALFRED AST60 in the time period analyzed. Lack of correlation was found only against *A. baumannii* strain 35/612: the instrument detected the resistant to CLX and not to DECS and Et-OH.

Discussion and Conclusions

Disinfectants and antiseptics are included in the large family of anti-infective, synthetic or natural origin, substances, able to eliminate different types of microorganisms. In response to outbreaks, especially in hospital environment, of etiologic agents resistant to traditionally in use disinfectants, it seems clear that the approach to anti-infective problem should follows several guidelines: prophylaxis of infectious diseases, use of new antibiotics and rational use of new disinfectants (4). In this regard, it was considered interesting to compare the antibacterial activity of four different chemical

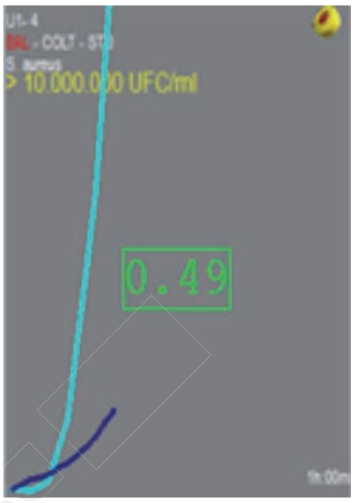

Figure 1. Alfred AST60 growth curve of *S. aureus* ATCC 29213 0.5 McFarland witness.

Table 1. Filtration tests results against ATCC strains.

Reference strains (ATCC)	IP	EtOH	CLX	DECS
E. coli ATCC 25922	AC	C	AC	AC
P. aeruginosa ATCC 27853	AC	AC	AC	AC
E. faecalis ATCC 29212	AC	AC	AC	AC
S. aureus ATCC 29213	AC	AC	AC	AC

AC: lack of growth; C: growth.

Table 2. Alfred AST60 tests results against ATCC strains.

Substance dilutions, % strain	*E. coli*	*P. aeruginosa*	*E. faecalis*	*S. aureus*
IP 25	AC	AC	AC	AC
IP 12.5	AC	AC	AC	AC
IP 5	C	C	KLL	BT-ST
IP 2.5	C	C	C	C
EtOH 50	AC	AC	AC	AC
EtOH 25	AC	AC	KLL	AC
EtOH 12.5	BT-ST	C	BT-ST	BT-ST
CLX 2.5	AC	AC	AC	KLL
CLX 2	AC	AC	AC	KLL
CLX 1.25	AC	AC	C	AC
DECS 5	AC	AC	AC	AC
DECS 2.5	AC	KLL	AC	KLL
DECS 1.25	KLL	C	BT-ST	BT-ST 60/ KLL 120

AC: lack of growth; C: growth; BT-ST: bacteriostatic effect; KLL: killing.

groups antiseptic-disinfectant molecules. We weight reference method, membrane filtration, up against automated Light Scattering one, with the use of ALFRED AST60 analyzer (Alifax SpA). The hypothesis is that this instrument can be also adapted to the *in vitro* evaluation of antiseptics activity. The technology used by this apparatus is optical scattering or spread or dispersion. The comparison tests between membrane filtration method and instrumentation ALFRED 60AST, gave concordant results. Overall ALFRED AST60 instrumentation exhibits results correlation in samples in which it is recorded plate growth; while it does not show up in the plate where there was a bacteriostatic effect. The concentrations of four different products tested with the filtration method were 50, 25 and 12.5%. The CMB values obtained with ALFRED 60AST assays against MDR clinical isolates have shown bactericidal effect of IP and IS (at 2.5% concentration of use). EtOH performs bactericidal effect (25%) of *E. coli*, *E. faecium*, *K. pneumoniae* and *P. aeruginosa* and bacteriostatic reaction on *A. baumannii* (at the concentration of use of 25%). CLX exhibited bactericidal power against MRSA and of all Gram-negative bacteria tested. It is noted that 1/3 strains of MRSA are resistant to EtOH and 1/3 strains of *E. faecium* showed resistance against CLX. More and more current and widespread is the indication for use in prophylaxis of antiseptics, even in the topical treatment skin, soft tissue and oral cavity chronic infections. The analyzer ALFRED 60AST can be appropriately adapted to *in vitro* evaluation of antiseptics, representing a valuable aid in the periodic monitoring of their activities and the prior assessment of sensitivity in case of therapeutic use. Though preliminary, the study confirms the existence of bacteria resistant to alcohols and biguanides, and it emphasizes the opportunity to verify their *in vitro* sensitivity profile.

Table 3. Alfred AST60 tests results against clinical isolates.

Clinical isolates	IP	EtOH	CLX	DECS
E. coli ESßL (4)	AC	AC	AC	AC
MRSA (3)	AC	C	KLL	AC
E. faecium (2)	AC	AC	C	AC
A. baumannii (3)	AC	KLL	KLL	AC
P. aeruginosa (2)	AC	AC	AC	AC
K. pneumoniae CP (3)	AC	AC	AC	AC

AC: lack of growth; C: growth; KLL: killing.

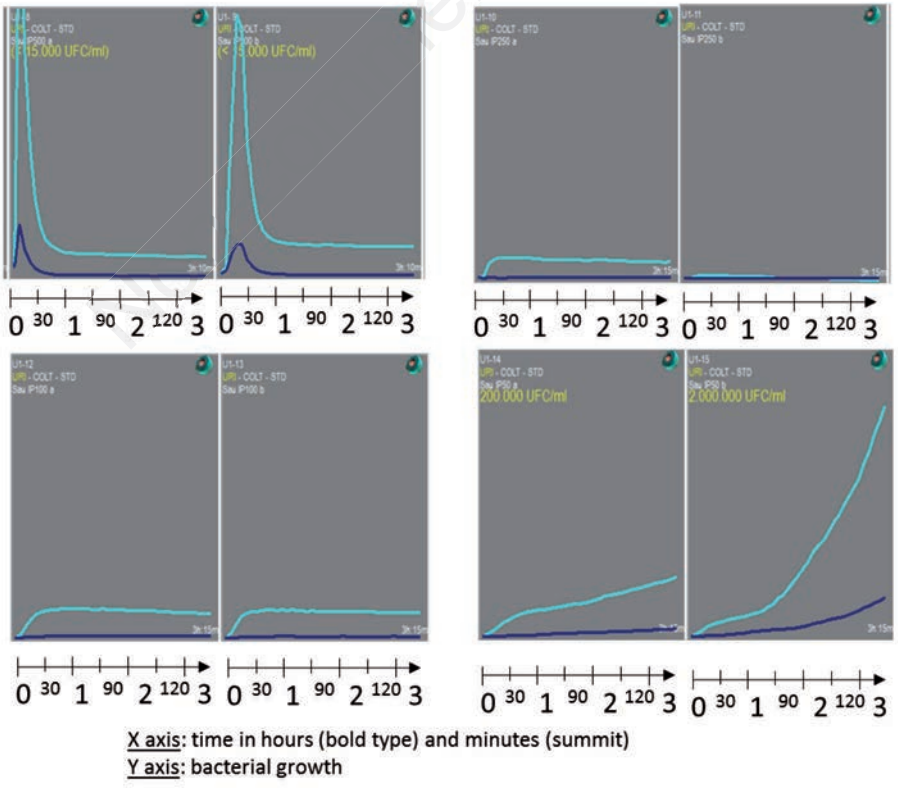

Figure 2. Alfred AST60 growth curve of *S. aureus* ATCC 29213 against IP: It is legitimate to point out the instrument signal disturbance at the beginning of the first two graphics due to the staining of the suspension, given by the Iodopovidone.

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