

An exception to the rule “no association between antibiotic resistance and decreased disinfectant microbicidal efficacy”: Orthophthalaldehyde (OPA) and *Pseudomonas aeruginosa* isolated from ICU and paraplegic patients

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Keywords

Bactericidal effect • OPA • *P. aeruginosa* • Antibiotic-resistance

Summary

Background. Antibiotic resistance and decreased susceptibility to disinfectants are not usually associated in microorganisms, but we have found an exception to this rule: *P. aeruginosa* versus orthophthalaldehyde (OPA).

Methods. Bactericidal effect of OPA was measured at 10 minutes on endodony files contaminated with an ATCC strain (control) or 206 strains of *P. aeruginosa* recently isolated from 206 ICU and paraplegic patients in a tertiary university hospital, in two consecutive years.

Results. Differences in bactericidal effect of OPA were found between the strains isolated each year (decreased susceptibility in the first period), but in both years the statistical differences ($p < 0.05$) were maintained according to whether the strains were “susceptible” to antibiotics, “resistant” (to one family of antibiotics) or “multi-resistant” (resistant to more than one family of antibiotics), exhibiting a reduction in their OPA susceptibility in parallel to an increase of their antibiotic resistance. In con-

trast, there were no differences depending on the type of sample (sputum, urine, faeces, pharynx) or of patient (paraplegic or ICU: adult, newborn, burn). Finally we selected 15 strains with an OPA effect below $3.5 \log_{10}$ at 10 minutes and repeated the study with an OPA exposure of 15 minutes. In these conditions OPA showed a total bactericidal effect on these *P. aeruginosa* strains.

Conclusions. There was an association between antibiotic resistance and decreased OPA susceptibility. This normally does not require an increase in disinfection time, but, for endoscope disinfection or instruments from colonized/infected patients with resistant/multiresistant *P. aeruginosa*, we consider it better to use 15 min of OPA. Regular tests (e.g., once every 12 months) with germ-carriers, should be performed to assess ecological changes in susceptibility to high level disinfectants and must include not only ATCC strains, but also recently isolated microorganisms with different antibiotic sensitivities (susceptible, resistant and multi-resistant).

Introduction

Globally, there is no association between antibiotic resistance and decreased susceptibility to disinfectants, but this may not be so with some disinfectants and microorganisms [1, 2]. Russell [3] states that low levels of biocide resistance can be detected and questions if this increased resistance could be related to domestic disinfectant use. This would first allow for a generalized selection of organisms that are more resistant to disinfectants, and these could colonize people through water and food [4]; second, an intestinal selection could occur through the use of antimicrobials. This would be better detected in the clinical setting, particularly in ICUs, where antibiotic consumption is greater [5, 6].

Complete resistance (no efficacy) to disinfectants is rare and we can only demonstrate a “decreased susceptibility” to these products. This lower susceptibility is normally related to cell wall alterations (the loss of porins

or the presence of incomplete lipopolysaccharides [7]) with changes in their permeability, or to an increase in the mechanisms for expulsion of products that are harmful to the bacterium [8-10]. This decreased susceptibility is not specific to disinfectants or antibiotics [11], since both can be associated in some bacteria.

In an earlier work [12] we observed a minor susceptibility of *P. aeruginosa* to orthophthalaldehyde (OPA) that was related to antibiotic resistance; strains exhibiting resistance to one or more than one antibiotic family (“resistant or multi-resistant” strains) also had a decreased susceptibility to the disinfectant. But our previous results [12] also have demonstrated an increase of OPA effectivity without changes in antibiotic resistance, after aging the same strains of *P. aeruginosa*. This indicated that the two mechanisms were independent, and were only “concurrent”, in recently isolated microorganisms from the patients.

Last, *P. aeruginosa* is a very frequent microorganism in infections of ICU or paraplegic patients and it normally, shows resistance to antibiotics. Moreover, these bacteria produce reiterated contamination of endoscopes, probably due to their ability to form a biofilm, and this can produce failures in high level disinfection processes [13-16]. Consequently, *P. aeruginosa* from these patients can be a good model of interaction between inadequate disinfection and antibiotic resistance.

The objective of this study was to confirm the association between a lower susceptibility to OPA and greater antibiotic resistance in *P. aeruginosa* with a larger sample of microorganisms, including not only resistant [12], but also susceptible strains, and, as well, to study if these conditions are associated to the type of sample used (source of microorganism, type of patient) or to resistance to a specific antibiotic.

Materials and methods

MATERIALS

- Disinfectants: OPA: 0.55% orthophthalaldehyde (Johnson & Johnson, Irvine, CA, USA).
- Microorganisms: *P. aeruginosa* ATCC 27853, and 206 *P. aeruginosa* strains isolated from patients during two consecutive years.
- Germ-carrier: Number 25, endodocny files (difficult to disinfect; these pieces, used in endodocny work, have a rough metallic surface with a rough plastic end).
- Glass beads (0.25 mm in diameter).
- Inhibitor of disinfectant action: Todd Hewitt broth (Difco) plus 6% (w/v) Tween 80, 0.5% (w/v) sodium bisulfite, and 0.5% (w/v) sodium thiosulfate.
- All culture media and Tween 80 used in this research were purchased from the Madrid Autonomous University Foundation (*Fundación Universidad Autónoma de Madrid, FUAM*).

METHODS

During two consecutive years (2011-2012), 206 samples (103 per year) were isolated from 206 different patients admitted to the different ICUs of Hospital La Paz (General, Burn and Newborn Units), as well as from urine or decubitus ulcer samples taken within the first three months after injury from paraplegic patients admitted to hospital. The sample (only one per patient) was the first *P. aeruginosa* isolated during their hospital stay, and the bacterial antibiotic susceptibility or resistance was recorded.

In the first week after isolation of these strains, we studied the bactericidal effect of OPA, using a method described in earlier studies [2, 12, 17] and summarized below:

DETERMINATION OF BACTERICIDAL EFFECT OF A DISINFECTANT EMPLOYING A METAL/PLASTIC GERM-CARRIER (NUMBER 25 ENDODONCY FILES)

Endodocny files (an excellent model of rough-carrier), were contaminated with a suspension of one strain of

these *P. aeruginosa* (10^8 CFU/ml) by immersion for one hour before being left to dry (15 minutes) on a slanted sterile surface (Petri dish with no culture medium). After drying, the germ-carrier was placed in a tube with 7 ml of the disinfectant for 10 minutes. The carrier was then removed and placed in another tube containing 7 ml of inhibitor with 0.5 g glass beads (1 mm in diameter) and vortexed for one minute at 1000 rpm. Finally, 0.1 ml of the supernatant was cultured on Mueller-Hinton plates and incubated at 37°C for 24 hours in order to count the number of microorganisms surviving after exposure to the disinfectant. The CFU counted were compared with these obtained for the control (using the same method but introducing the germ-carrier in sterile distilled water instead of disinfectant). The assay was performed for all microorganisms described in the Materials section.

The cut point for considering "reduced OPA susceptibility" was a \log_{10} reduction of less than 3.5 [17] (equivalent to below 5 \log_{10} when using the test involved glass germ-carriers in the EN-test, as described elsewhere [12]). Both cut points (according to their test) indicate an inadequate disinfection.

Finally, of 15 randomized strains, among all those exhibiting a bactericidal effect of $< 3.5 \log_{10}$ after 10 minutes of OPA exposure, we repeated the test with a 15 minute exposure.

ANTIBIOTYPE

The antibiotype was obtained on the same day of the bactericidal effect with each *P. aeruginosa* strain. The method used was Kirby-Bauer. The cut point for being considered resistant was according to CLSI, 2007.

STATISTICAL ANALYSIS

For the statistical study, a $> 5.5 \log_{10}$ reduction (experiments in which there were no surviving *P. aeruginosa* CFU) was considered as "5.5" \log_{10} .

Demonstration of significant differences between OPA susceptibility (\log_{10} reduction) and antibiotic susceptibility according to type of microorganism source was done using an analysis of variance (ANOVA) or a Mann-Whitney U test and Kruskal-Wallis test, since nonparametric tests were used for all samples lacking a normal distribution.

Finally we have performed a multivariable analysis by logistic regression, taking as dependent variable the \log_{10} reduction of *P. aeruginosa* ($< 3.5 \log = 1$ and $\geq 3.5 = 0$) and as independent variables, type of patients, source of strains, year and antibiotype (classified into susceptible, resistant and multi-resistant or by specific antibiotics too).

Results

Patient distribution was: general ICU 75.7%, neonatal ICU 12.1%, burn unit 4.8% and paraplegics 6.8%.

The source of the 206 *P. aeruginosa* isolates from the 206 patients was as follows: pharynx or sputum 70.1%,

Tab. I. \log_{10} reduction of 207 *P. aeruginosa* (206, isolates from 206 patients and one ATCC strain), on germ-carrier, according to antibiotic resistance, after 10 minutes of exposure to OPA.

\log_{10} reduction by OPA				
	Mean	Standard Error	50-Percentile	p
Antibiotic susceptibility				
ATCC*	5.5	0**	5.5	
				< 0.01
Susceptible	4.9	1.1	5.5	
				< 0.01
Resistant	4.46	0.97	4.6	
				< 0.01
Multi resistant	3.97	1.1	3.88	

* Repetition of 10 tests with the same ATCC *P. aeruginosa*: control strain; ** There was no variability among the different experiments, because > 5.5 \log_{10} was always obtained (recorded in the statistical analysis as 5.5).

Tab. II. \log_{10} reduction of 207 *P. aeruginosa* (206, isolates from 206 patients and one ATCC strain), on germ-carrier, after 10 minutes of exposure to OPA, according to the origin of the bacteria.

\log_{10} reduction by OPA				
	Mean	Standard Error	50-Percentile	p
Type of sample				
Pharynx/sputum	4.2	1.1	4.1	
				NS
Urine/faeces	4.3	1.2	4.4	
				NS
Nasal	4.4	1.1	4.7	
				NS
Other	5.1	0.75	4.8	
Type of patient				
General ICU	4.3	1.1	4.4	
				NS
Neonate ICU	4.4	1.1	4.9	
				NS
Burn unit	4.6	0.75	4.5	
				NS
Paraplegics	4.1	1.05	3.95	

urine or faeces 15%, nose 8.9%, and other 6% (i.e., burn, decubitus ulcer or central venous catheter insertion site). These 206 *P. aeruginosa* were distributed into three groups based on antibiotic susceptibility: “susceptible” (only natural resistance) 21.8%, “resistant” (to one antibiotic family – independently of what it was – in addition to natural resistance) 34%, and “multi-resistant” (resistant to two or more antibiotic families, in addition to natural resistance) 44.2%.

The logarithmic reduction originated by OPA on *P. aeruginosa* strains was distributed according to the above variables. Last we included the year of diagnosis.

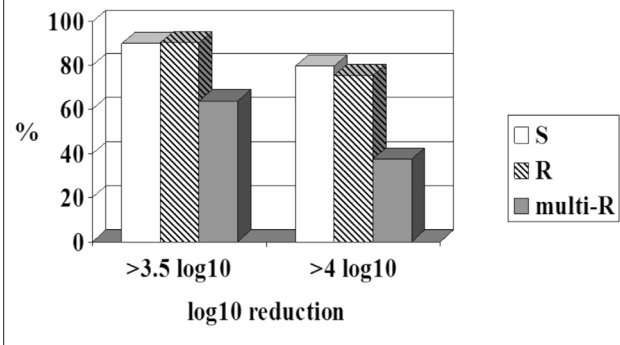
Tables I and II showed that antibiotic susceptibility is the only parameter that significantly ($p < 0.001$) differentiated the *P. aeruginosa* strains. Moreover, the ATCC *P. aeruginosa* strain was also different from the antibiotic-susceptible strains, because the ATCC strain was fully susceptible to OPA (0 survivors in all experiments).

On the one hand, Figure 1 shows the overall frequency of strains on which OPA had a bactericidal ef-

fect > 3.5 \log_{10} (the optimum threshold in this test) or > 4 \log_{10} (considered here as “great efficacy”). It can be seen that 90% of antibiotic susceptible or resistant *P. aeruginosa* strains reached the 3.5 \log_{10} reduction with OPA while this product only had a similar bactericidal level in two-thirds of the multi-resistant strains, with significant differences between the susceptible, resistant and multi-resistant strains. However, great efficacy (> 4 \log_{10} reduction), was still achieved in 79.5% of the susceptible and 75.4% of the resistant strains but in only one-third of the multi-resistant strains. In both thresholds, there were significant differences between the OPA’s effect against susceptible or resistant strains versus the effect in multi-resistant strains.

In 15 randomized strains (among all those exhibiting a bactericidal effect of < 3.5 \log_{10} after 10 minutes) were exposed to OPA during 15 minutes. We obtained a complete destruction of all the microorganisms, suggesting that even in the worst case scenario (and using more resistant strains and complex instruments), a complete

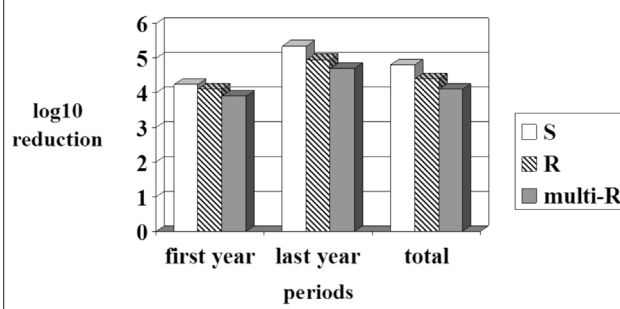
Fig. 1. Bactericidal effect of OPA against 206 *P. aeruginosa* strains, by antibiotic type.



destruction of the microbial inocula could be achieved by simply prolonging exposure by 5 minutes.

Last, Figure 2 shows the existence of differences between the two studied years. Thus, in the first, *P. aeruginosa* isolates were on average more resistant to OPA than in the second year, but the differences between susceptible, resistant and multi-resistant strains were maintained in both periods. When all strains were jointly assessed, (or when the strains from the first year were considered only) a statistical association was found between resistance to imipenem or two aminoglycosides (gentamycin and tobramycin) and a bactericidal effect below the optimum threshold of 3.5 log₁₀. However this did not occur in the second year, since the *P. aeruginosa* isolates were much more susceptible to OPA, and only a small proportion fell below the threshold, whereas the resistance to these 3 antibiotics remained virtually unchanged. Therefore, this antibiotic resistance to imipenem and aminoglycosides cannot be generalized as a predictive antibiotic marker for a decreased/weaker bactericidal effect. No other antibiotic was associated with a decreased bactericidal effect (taking strains resistant to 1-5 antibiotics in all possible combinations, for example: amikacin + imipenem, amikacin + ceftazidime, amikacin + fosfomycin, amikacin + imipenem + ceftazidime + fosfomycin, etc.). Multivariable logistic regression, taking as dependent variable the log₁₀ reduction of *P. aeruginosa* and as in-

Fig. 2. Bactericidal effect of OPA against 206 *P. aeruginosa* strains, by time and antibiotic type.



dependent variables, type of patients, source of strains, year and antibiotic type, did not show a good fit.

Discussion

The availability of a large number of samples with different antibiotic susceptibilities allowed us to adequately assess the association between *P. aeruginosa* antibiotic resistance and decreased susceptibility to a disinfectant (in this case OPA) from a statistical viewpoint (N-dependent [18]).

ATCC strains are helpful in homogenizing the results obtained in different laboratories, but they are not good predictors of the true performance of a disinfectant in the clinical setting, since these strains frequently show a complete destruction of the inocula (i.e., maximum susceptibility), unlike autoctonous strains [12, 17] (Tab. I). These considerations indicate a need to add to tests using ATCC strains other tests with strains that have been recently isolated from patients (better in the first week, with no more than a single culture passage [12]). When possible, not only antibiotic-susceptible but also resistant and multi-resistant strains should be included in these tests.

It is noteworthy that changes were also seen when the bactericidal effects were compared using microorganisms from both consecutive years (mean log₁₀ reduction: 3.6 ± 0.7 in the first, versus 4.8 ± 0.8 in the second year; p < 0.01), and these differences persisted after stratification according to antibiotic susceptibility (Fig. 2). Another difference was also noted in these two years: the lack of an association between resistance to imipenem, gentamycin and tobramycin, and a bactericidal effect below the optimum threshold of 3.5 log₁₀ in the second year. That does not allow generalization of these markers as indicators for a need to increase the OPA disinfection time.

These differences are therefore probably not due to methodological changes (method, laboratory, microbiologist, control strains, inhibitor of disinfection, etc.) because they were the same in all experiments in both periods. The patient-sources for *P. aeruginosa* were similar too. We therefore believe that the reason could be the modifications in the dominant strains in the hospital caused by antibiotic use or environmental changes (home disinfectants or antibiotherapy). This should be taken into account, and routine studies of the bactericidal efficacy of disinfectants used should be conducted to assess the need to extend disinfectant exposure time.

Implications in daily practice:

- A warning of "*P. aeruginosa* R or multi-R" should be included in the medical records of all patients colonized or infected by these microorganisms, so that the reusable instruments are disinfected with an increased OPA exposure time.
- Since overall almost a third of multi-resistant strains experience a bactericidal effect from OPA that is lower than 3.5 log₁₀ (considered the threshold value of the test [12]), it could be advisable to prolong ex-

posure time to the disinfectant from 10 to 15 minutes, as with this latter time even the most resistant strains are totally destroyed. This exposure-time in endoscope disinfection with OPA is closer to USA recommendations (12 min) than European ones (5 min) [19].

- The effectiveness of OPA disinfection should be compared between instruments used in patients with resistant or multi-resistant *P. aeruginosa*. This is especially important for endoscopes, since the persistence of *P. aeruginosa*, after disinfection, means an increased risk of infection for the next patient undergoing this technique. The ideal sample in these cases is the rinse water of the equipment, after disinfection with OPA.

However, the risk of disinfection failure is very low in any case, since if thorough washing with an enzymatic detergent is carried out prior to disinfection, the inoculum to be dealt with by the disinfectant is at the most 3.1-4.3 log₁₀ on average in the case of endoscopes [20, 21]; moreover, after disinfection, supplementary rinsing will also help remove any remaining microorganisms. Thus, at the end of the process no residual microorganisms are likely to be isolated, as seen in the serial controls we perform on endoscopy equipment once a month in our hospital, even when disinfectants less potent than OPA were used [17]. However, it is necessary to maximize caution to ensure the high quality disinfection demanded by modern hospitals, and the conditions of the process should be adapted to the patients e.g. increase the disinfection time for a colonoscope, from a patient with antibiotic-resistant microbiota, to 15 min.

- We advise at least one study every 12 months using not only ATCC strains but also recently isolated bacteria on a complex germ-carrier (like an endodony file), to help evaluate the necessity of increasing disinfectant exposure time.

Limitations:

- This design only includes two years of *P. aeruginosa* sampling. It does not allow us to explain the cause of differences in OPA susceptibility when the patients and antibiotic use in these ICUs were similar. It would be interesting to study more years to understand the cause of these changes.
- Despite the large number of strains studied, our results did not obtain any antibiotype that would serve as a marker of reduced OPA efficacy. We only know that “resistant” or “multi-resistant” *P. aeruginosa* can produce a failure in disinfection when OPA is used, which is less useful in daily practice.
- We have not studied patients at digestive care units. This can be a problem, given that endoscopic techniques are used more frequently to treat these patients. However, we have found that the selection of resistant or multi-resistant *P. aeruginosa* among them is very low because they are less frequently treated with antibiotics than patients in UVIs, burned or paraplegic patients.

- In order to increase the homogeneity of the sample, only the first strain of *P. aeruginosa* isolated in each patient was included in the study. Unfortunately, this reduces our ability to study if the evolution of antibiotic resistance affects the susceptibility of microorganism to OPA.

Conclusions

An association exists between antibiotic resistance and decreased susceptibility to OPA for *P. aeruginosa*, however, in practical terms the reduction in efficacy normally does not imply an increase in disinfection time, except in flexible endoscope disinfection, where 15 min is advisable, regardless of their colonization or infection with *P. aeruginosa*.

As a precaution, reusable instruments from patients colonized or infected with resistant or multi-resistant *P. aeruginosa* should be treated with 15 minutes of OPA. This must be recorded in the clinical history of these patients.

In the cases of colonization or infection by resistant or multi-resistant *P. aeruginosa*, the efficacy of disinfection should be evaluated (for example, by sending to the Microbiology laboratory a sample of the rinse water of the endoscope after disinfection with OPA).

Regular tests (e.g., once every 12 months) should be performed to assess ecological changes in susceptibility to OPA by *P. aeruginosa* colonizing or infecting patients, in order to detect changes in strain susceptibility and to be able to recommend an increase in disinfectant time (e.g. 5-10 to 15 minutes) when necessary.

Any evaluation of the efficacy of OPA should include not only ATCC strains but also recently isolated autochthonous microorganisms with different antibiotic sensitivities (susceptible, resistant and multi-resistant), since such characteristics may condition reduced product susceptibility.

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Authors' contributions

RH conceived and designed the research. IH, RH and MJV performed the microbiological and the statistical analysis. RH wrote the manuscript. All authors revised and approved the final manuscript.

Revision of the test by a native English speaker (C. Warren).

References

- [1] Herruzo I, Herruzo R, Vizcaino MJ. *It's possible to predict a decreased bactericidal effect of biocides, through antibiotic re-*

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- sistance in ICU: Study using a large sample of bacteria and multivariate analysis. *Adv Infect Dis* 2015;5:73-80.
- [2] Herruzo R, Vizcaino MJ, Herruzo I. Can the antibiotic resistance of a microorganism predict decreased bactericidal efficacy of disinfectants? Application to OPA and other products. *EJC-MID* 2009;28:539-41.
- [3] Russell AD. Do biocides select for antibiotic resistance? *J Pharm Pharmacol* 2000;92:227-33.
- [4] Higgins CS, Murtough SM, Williamson E, Hiom SJ, Payne DJ, Russell AD, Walsh T. Resistance to antibiotics and biocides among non-fermenting Gram-negative bacteria. *Clin Microbiol Infect* 2001;6:308-15.
- [5] Albrich WC, Angstwurm M, Barder L, Gartner R. Drug resistance in intensive care units. *Infection*. 1999;27(Suppl):19S-23.
- [6] Roberts SA, Findlay R, Lang SD. Investigation of an outbreak of multidrug resistant *Acinetobacter baumannii* in an intensive care burns unit. *J Hosp Infect* 2001;48:228-32.
- [7] Russell AD, Furr JR. Susceptibility of porin and lipopolysaccharide deficient strains of *Escherichia coli* to some antiseptics and disinfectants. *J Hosp Infect* 1986;8:47-56.
- [8] Poole K, Krebes K, McNally C, Neshat S. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *J Bacteriol* 1993;175:7363-7372
- [9] Alonso A, Campanario E, Martinez JL. Emergence of multidrug-resistant mutants is increased under antibiotic selective pressure in *Pseudomonas aeruginosa*. *Microbiology-UK* 1999;145:2857-62.
- [10] Alekshun MN, Levy SB. The *mar regulon*: multiple resistance to antibiotics and other toxic chemicals. *Trends Microbiol* 1999;7:410-3.
- [11] Cremieux A, Freney J, Davin-Regli A. Methods for testing disinfectants. In: Block SS, ed. *Disinfection, Sterilization and Preservation*. Fifth ed. Philadelphia: Lippincott Williams and Wilkins 2000. pp. 1305-27.
- [12] Herruzo-Cabrera R, Vizcaino-Alcaide MJ, Fernández-Aceñero MJ. Influence of laboratory adaptation of test strains (example *P. aeruginosa*) in the evaluation of the antimicrobial efficacy of Orthophthalaldehyde (OPA). *J Hosp Infect* 2004;57:217-22.
- [13] Srinivasan A, Wolfenden LL, Song X, Mackie K, Hartsell TL, Jones HD, Diette GB, Orens JB, Yung RC, Ross TL, Merz W, Scheel PJ, Haponik EF, Perl TM. An outbreak of *Pseudomonas aeruginosa* infection associated with flexible bronchoscopes. *N Engl J Med* 2003;348:221-7.
- [14] Kovaleva J, Peters FTM, van der Mei HC, Degener JE. Transmission of infection by flexible gastrointestinal endoscopy and bronchoscopy. *Clin Microbiol Rev* 2013;26:231-54.
- [15] Harmsen M, Yang L, Pamp SJ, Tolker-Nielsen T. An update on *Pseudomonas aeruginosa* biofilm formation, tolerance and dispersal. *FEMS Immunol Med Microbiol* 2010;59:253-68.
- [16] Donskey CJ. Does improving surface cleaning and disinfection reduce health care-associated infections? *Am J Infect Control* 2013;41:S12-9
- [17] Herruzo-Cabrera R, Vizcaino-Alcaide MJ, Rodriguez J. Comparison of the microbicidal efficacy on germ carriers of several tertiary amine compounds with ortho-phthalaldehyde and Perasafe. *J. Hosp. Infect* 2006;63:73-8.
- [18] Carrasco JL. *El método estadístico en la investigación médica*. Madrid: Ed Ciencia 3 1989.
- [19] Rutala W, Weber DJ and the Healthcare Infection Control Practices Advisory Committee (HICPAC). *Guideline for Disinfection and Sterilization in Healthcare Facilities*. 2008.
- [20] Alfa M, Sitter DL. In-hospital evaluation of orthophthalaldehyde as a high level disinfectant for flexible endoscopes. *J Hosp Infect* 1994;26:15-26.
- [21] Hernandez A, Matro E, Puzo C, Mata L, Burgues C, Vazquez N, Castella J, Ausina V. In-use evaluation of Perasafe compared with Cidex in fiberoptic bronchoscope disinfection. *J Hosp Infect* 2003;54:46-51.

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