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An in vitro test for the evaluation of the efficacy of disinfectants

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Summary

The paper presents a method for the in vitro evaluation of the effectiveness of disinfectants. It is a capacity test performed in hard water with or without horse serum. Some results are presented showing the ability of growth in disinfectant solution.

Introduction

It was usual some years ago to make a firm distinction between disinfectants and antiseptics. It was then accepted, and indeed it is still accepted in some countries, that the former category are agents for disinfecting the environment (water, air, floor and wall surfaces, furniture, equipment, etc.) whereas the latter are designed specifically for external application to skin and mucosal body surfaces.

In practice today, however, both these definitions of antimicrobial action, often refer to the same active agent which may be termed 'antiseptic' or 'disinfectant' depending largely upon the concentrations at which it is used or the purposes for which it is required. In several European countries, notably Switzerland and Belgium, it is now common to restrict the term 'antiseptic' to purely a curative role or function and then to further define the term 'disinfectant' depending on its specific use. Thus a disinfectant may be categorized as:

(A) *Environmental disinfectants*—e.g. surgical instrument disinfectants; disinfectants for hospital walls and floors; industrial (dairy or pharmaceutical) disinfectants; and disinfectants for infected dressings and excreta; or

(B) *Disinfectants for application to body surfaces*—e.g. hand disinfectants; disinfectants for general hand hygiene; pre-operative disinfection of operators' hands; pre-operative skin disinfection of patients; general skin disinfection prior to an injection or other procedure when the cutaneous barrier is penetrated; mucosal

surface disinfectants; and wound disinfectants.

Categories A and B and their respective examples are largely self explanatory; a subtle distinction must be made, however, between the first two examples in category B. Hand disinfectants are commonly used in human and veterinary medicine to disinfect the hands after possible contamination by micro-organisms; disinfectants for general hand hygiene are used to disinfect hands which are not especially contaminated but are required to be kept relatively free from microbial contamination over a short period.

The division of usage of disinfectants into these categories and sub-categories greatly helps to define the efficacy and the safety that is to be required of the agent in any specific use.

Disinfection is a procedure used to prevent the transmission of contaminating micro-organisms. The emergence of organisms resistant to antibiotics (Bauernfeind, 1976) has given a new dimension to this prophylactic use of disinfectants. Since it is clear that disinfectants must act irrespective of the physiological state of the micro-organism (latent, exponential or stationary phase of growth), the disinfectant should be bactericidal or microbiocidal rather than bacteriostatic or microbiostatic. Furthermore, to prevent bacterial transmission, it is optimal that the disinfectant exerts its killing effect in as short a time as is consistent with its intended use; obviously this killing time can vary according to the use of the disinfectant.

Other requirements for disinfectants may also be defined; antimicrobial action must be maintained in the presence of Ca^{2+} or Mg^{2+} in hard water, in the presence of organic matter or when in contact with soaps or detergents. With some disinfectants, for example, those used on hands or skin, a residual effect may also be required.

The evaluation of the efficacy of a disinfectant requires 3 distinct phases of experimentation. Firstly, it requires a preliminary experimental laboratory study of the antimicrobial properties of the disinfectant over a range of concentrations. It is necessary at this stage to standardize the strains of micro-organisms and the inoculum. This in vitro study will therefore provide some estimate of the agent's activity against the selected strains of organisms.

The second phase of the examination is a laboratory study in which the disinfectant is screened for specific activity by tests which relate to the suitability of the disinfectant for specific purposes (e.g. disinfection of surfaces, excreta, dressings, hands, etc.). These tests are also performed with identified and categorized strains of micro-organisms.

The final stage of the examination of the disinfectant is a 'field-trial' of its activity. This permits further evaluation of the results of the laboratory studies; it identifies the organisms selectively inhibited by the agent and in doing so, gives some indication of the physiological properties of these strains.

It is with regard to the preliminary laboratory study of phase one of the investigation that this present report is concerned. The testing procedure is described and discussed and some experimental results are presented.

Materials and Methods

Reagents

(1) Test organisms: *Staphylococcus aureus* ATCC 6538; *Pseudomonas aeruginosa* ATCC 15442; *Klebsiella pneumoniae* ATCC 10031; and *Escherichia coli* ATCC 11229.

In some cases *Salmonella typhi* ATCC 6539 for the disinfection in dairy and agricultural industry.

(2) Hard water: for the dilution of the products we used hard water prepared by adding 402 mg of $CaCl_2 \cdot 2\,H_2O$ and 139 mg $MgCl_2 \cdot 6\,H_2O$ to 1 liter of double-distilled water.

(3) Serum: sterile horse serum (Institut Pasteur du Brabant), inactivated by 30 min incubation at 56°C, was used to test the influence of organic material on the efficacy of the disinfectants.

(4) Culture media: H.I.A., heart infusion agar (Difco); B.H.I., brain heart infusion (Difco); Letheen agar (Difco); Neutralizing liquid medium (neutralizing solution), i.e. peptamine (10.0 g), beef extract (5.0 g), lecithin (3.0 g), Tween 80 (30.0 g), L-histidine (1.0 g), sodium thiosulfate (5.0 g), sodium chloride (5.0 g) and water (1000 ml). Autoclave at 121°C for 15 min. The efficacy of the neutralizing solution was established in a preliminary test in each case.

(5) Diluent for the bacterial count: phosphate salt peptone buffer at pH 7, i.e. monopotassium phosphate (3.56 g), disodium phosphate $\cdot 2H_2O$ (7.23 g), sodium chloride (4.30 g), peptone (meat or casein) (1.00 g) and water (1000 ml). Autoclave at 121°C for 15 min.

Apparatus

(1) Tubes 150 × 18 mm with round bottom. (2) Disposable sterile petri dishes, diameter 90 mm. (3) Vortex mixer.

Methods

(1) Maintenance of test culture: stock cultures were maintained at 4°C on H.I.A. slants. Continuous cultures were made by daily transfer on H.I.A. slants and incubation at 35 ± 2°C. Trials were performed only after 3 subcultures.

(2) Preparation of the bacterial suspension: 100 ml of B.H.I. were inoculated and incubated for 18 h at 35 ± 2°C. Before the test the suspension was shaken for 1 min with sterile glass balls and a magnetic stirrer. The number of viable micro-organisms in the suspension was determined by plate count and ranged between 5×10^8 to 5×10^9 organisms/ml. If this was not the case, the test was considered to be invalid.

(3) Preparation of the disinfectant solution: immediately before the trial the disinfectant was diluted to the test concentration in sterile hard water. If the dilution prescribed using another diluent (for example, distilled water), a test using this diluent was carried out.

(4) Test procedure (Fig. 1): the test was carried out at 20 ± 1°C and all reagents were prewarmed for at least 30 min. The mixtures should have been shaken for about 5 s on a vortex mixer after each manipulation.

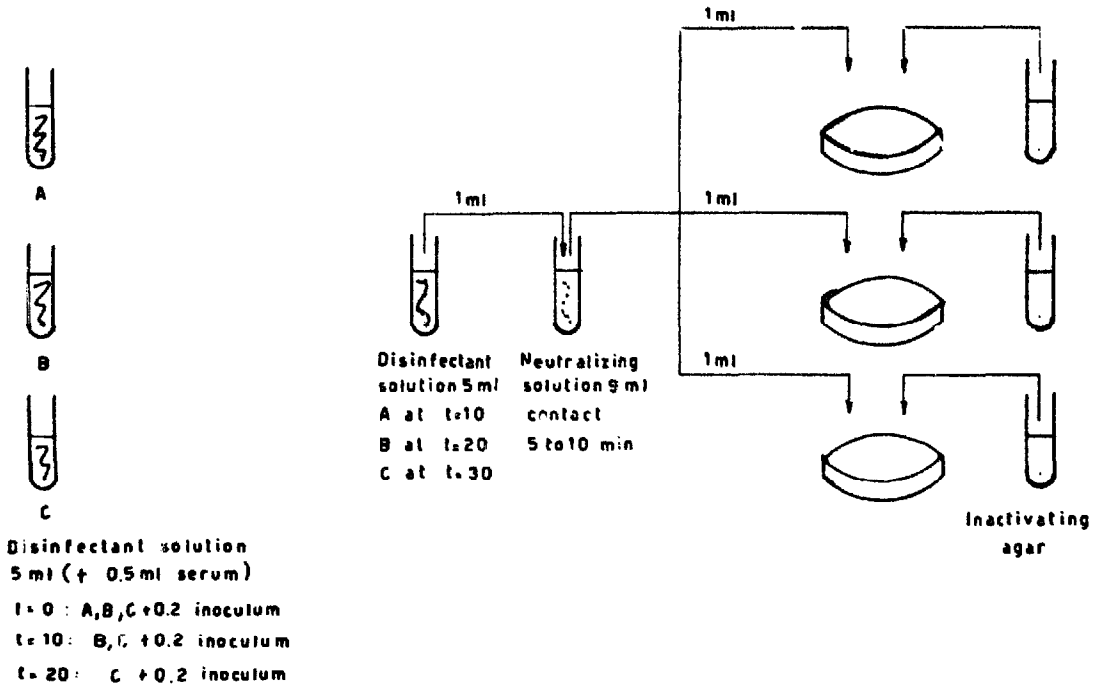

Fig. 1. Test procedure.

(a) Procedure in the absence of serum. (i) At time zero 0.2 ml of the inoculum was added to 5 ml of disinfectant solution. The mixture was shaken for about 5 s and placed back in the water bath. The inoculum size at that stage was between 10^8 and 10^9 bacteria in 5.2 ml of disinfectant solution, say about 1.9×10^7 to 1.9×10^8 bacteria per ml (N). After 10 min $\pm$ 5 s 1 ml was transferred into 9.0 ml of neutralizing solution and subculture as described under (5).

(ii) A second tube was taken and 0.2 ml of the inoculum added to 5 ml of disinfectant solution. This mixture was shaken for about 5 s and then placed back into the water bath. After 10 min $\pm$ 5 s 0.2 ml of the inoculum was again added to the mixture, after which it was shaken for about 5 s and placed back in the water bath. After a second contact time of 10 min $\pm$ 5 s 1 ml of the mixture was transferred into 9.0 ml of neutralizing solution and subculture as described under (5).

(iii) A third tube was taken and 0.2 ml of the inoculum added to 5 ml of disinfectant solution. This mixture was shaken for about 5 s and replaced in the water bath. After 10 min $\pm$ 5 s 0.2 ml of the inoculum was again added to the mixture, it was shaken for about 5 s and placed back into the water bath. After a second contact time of 10 min $\pm$ 5 s 0.2 ml of the inoculum was added the mixture shaken for about 5 s and then placed back into the water bath. After a last contact time of 10 min $\pm$ 5 s 1 ml of the mixture was transferred into 9.0 ml of neutralizing solution and subculture as prescribed under (5).

(b) Procedure in the presence of serum. The whole procedure was repeated with the addition of 10% (0.5 ml) of decomplemented sterile horse serum. At time zero,

0.2 ml of the inoculum was added to the mixture of 5 ml disinfectant solution and 0.5 ml of serum. The inoculum at that stage was between 10^8 and 10^9 in 5.7 ml of disinfectant solution, say about 1.7×10^7 to 1.7×10^8 bacteria per ml (N').

(5) Subculture: after a contact time of 5–10 min 1.0 ml samples of the undiluted neutralized mixture were mixed with 20 ml of letheen agar, melted and cooled to 45°C, and eventually supplemented with neutralizers suitable for the disinfectant under test. All inoculations were carried out in triplicate. The colony-forming units (n or n') were counted after 48 h incubation at 35 ± 2°C.

(6) Calculation of the germicidal effect: the germicidal effect is the difference expressed as logarithm of the initial count N or N' minus the number of colony-forming units multiplied by 10 after the procedure with disinfectant—n without serum and n' in presence of serum.

$$\log N - \log(n \times 10) \geqslant 5$$

$$\log N' - \log(n' \times 10) \geqslant 5$$

This value must be equal to or greater than 5.

A disinfectant was considered as satisfactory when the bactericidal power of the dilution used gave a reduction of 5 log cycles of the initial number of bacteria after ten minutes. This effect must persist after two more additions of micro-organisms, i.e. after a total time of 30 min even in the presence of serum.

Assessment of the efficiency of neutralizers (Fig. 2)

(1) Preparation of the bacterial suspension: 100 ml of B.H.I. was inoculated and incubated for 18 h at 35 ± 2°C. Before the test the suspension was shaken for 1 min with sterile glass balls and a magnetic stirrer. This suspension was diluted 1/10,000 in phosphate salt peptone buffer. The number of viable micro-organisms was determined by plate count.

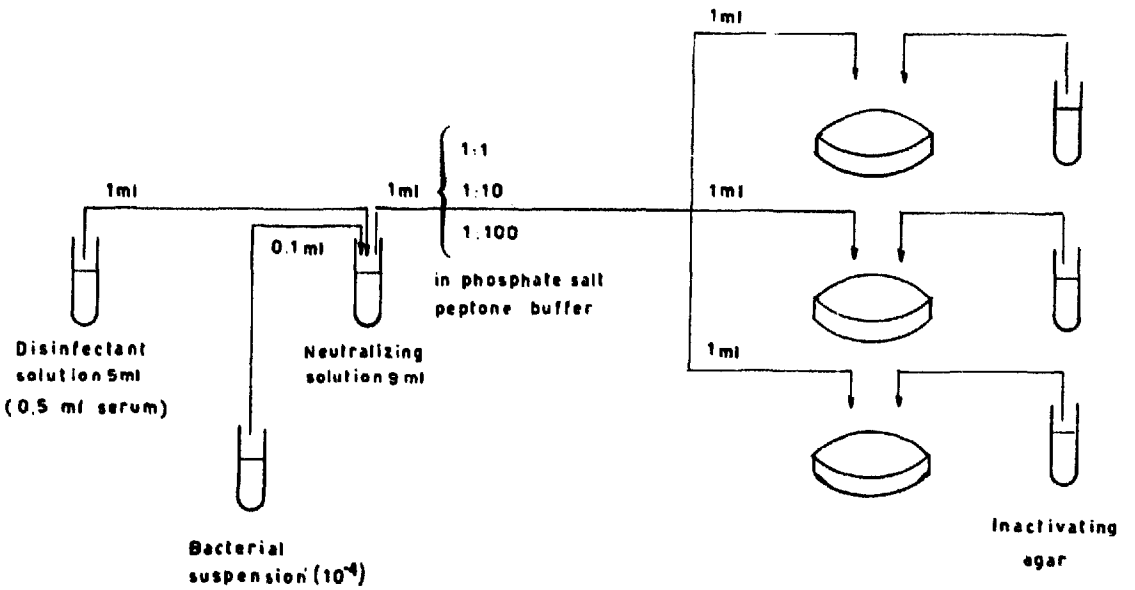

Fig. 2. Assessment of the efficiency of the neutralizers.

(2) Preparation of the disinfectant solution: the disinfectant was diluted to the highest test concentration in sterile hard water immediately before the test.

(3) Procedure: to a tube containing 9.0 ml of neutralizing solution 1.0 ml of the disinfectant solution was added. This mixture was shaken for about 5 s and placed back in the water bath. After a contact time of 5–10 min 0.1 ml of the prepared bacterial suspension was added. The mixture was shaken for about 5 s and placed back into the water bath. After a contact time of 5 min 1 ml of the undiluted mixture was transferred as well as 1 ml of the dilution 10^{-1}, 10^{-2} and 10^{-3} and mixed with 20 ml of letheen agar melted and cooled to 45°C, and combined with neutralizers suitable for the disinfectant under test. All inoculations were carried out in triplicate and incubated for 48 h at 35 ± 2°C. The same procedure was carried out using a 1-ml aliquot of a mixture containing 5 ml of the disinfectant concentration and 0.5 ml of sterile inactivated horse serum instead of the disinfectant solution. The neutralizers were satisfactory when there was no significant difference between the counts performed with the disinfectant and counts made with sterile water instead of the disinfectant.

Ability of micro-organisms to grow in the disinfectant solutions under testing often called a test of resistance to contamination

We restricted the choice of test organism to *Pseudomonas aeruginosa,* which is well known for its ability to survive and multiply in some disinfectant solutions. To verify the absence of multiplication of eventually surviving bacteria the tubes were taken without and with serum which received 3 inoculations of micro-organisms. After 10 days of storage at room temperature, 1.0 ml of this mixture was transferred to 9.0 ml of neutralizing solution and subculture. After a contact time of 5–10 min, 1-ml aliquots of the neutralized mixture were plated on letheen agar plates containing neutralizers suitable for the disinfectant under test. All inoculations were carried out in triplicate and incubated for 48 h at 35 ± 2°C. The number of bacteria must not be greater than that recovered in the previous capacity test.

A simplified scheme of our method has been described in a previous paper (Dony and Devleeschouwer, 1978). The reasons which led us to choose our test conditions such as the repeated additions of the inoculum, presence of hard water and organic matter, test organisms, have already been discussed in that publication. In this work we have given more emphasis to the experimental conditions which are given in detail and we will discuss results obtained by our method and the interest of the test of "resistance" to the contamination.

Results

Table 1 gives the results of our method for several products with 3 bacterial strains. In each case we present the name and concentration of the active molecule(s) in the product. Results are expressed in terms of satisfactory (S) when the germicidal effect is currently greater or equal to 5 log cycles, non-satisfactory (NS) when the reduction of the bacterial population is below 5 log cycles and limit (L) when, in a series of experiments, some of the results gives a bacterial reduction ranging between 4 and 5 log cycles.

TABLE 1

RESULTS OF THE EVALUATION OF THE EFFICACY OF DISINFECTANTS IN VITRO

Product	Concentration of the active molecule	*Staphyloccus aureus*		*Escherichia coli*		*Pseudomonas aeruginosa*		Resistance to contamination
		serum−	serum+	serum−	serum+	serum−	serum+	
A	Chloroxylenol 0.245%	S	S	–	–	S	S	↗
B	Chloroxylenol 0.147%	–	–	–	–	S	S	↗
C	Chloroxylenol 0.122%	S	S	S	S	L	S	–
D	Chloroxylenol 0.024%	L	NS	L	NS	NS	NS	–
E	Chloroxylenol 0.06% EDTA 0.082%	–	–	–	–	S	S	=
F	Alkyl and aryl phenols 2% commercial product	S	S	S	S	S	S	–
G	Alkyl and aryl phenols 0.5% commercial product	S	NS	–	–	S	S	=
H	Substituted phenols 0.16%	S	S	S	S	S	S	=
I	Chlorofen 0.008%	NS	NS	NS	NS	NS	NS	–
J	Chlorofen 0.04%	S	NS	S	NS	NS	NS	=or ↗
K	Chlorhexidine gluconate 0.05%	L	NS	S	L	L	NS	↘
L	Chlorhexidine gluconate 4%	S	S	S	S	S	S	–
M	Chlorhexidine 0.015% Cetrimide 0.15%	S	L	S	L	L	L	↘
N	Chlorhexidine 0.0075% Cetrimide 0.075%	L	L	S	L	S	NS	↘
O	Cetrimide 0.2%	S	S	S	S	S	S	–
P	n-Alkyl dimethyl benzyl ammonium chloride 0.15%+EDTA 0.02%	S	S	S	L	S	NS	↘
Q	Phenylmercuric borate 0.01%	S	S	L	NS	NS	NS	–
R	Formaldehyde 0.085%	NS	NS	NS	NS	NS	NS	↘
S	Formaldehyde 0.51%	NS	NS	–	–	NS	NS	↘
T	Formaldehyde 0.85%	NS	NS	–	–	S	S	↘
U	Aldehydes 0.147%	S	L	S	L	S	L	–

The last column gives the results of the test of resistance to the contamination. When the amount of bacteria increases after 10 days in the test tubes, this is denoted by an ascending arrow (↗). When the number of bacteria decreased, this is shown by a descending arrow (↘). Lastly, when there is no difference between the bacterial counts performed at the time of the test and 10 days later, this is shown by an equal symbol (=). In this latter situation, there could be no detectable survivors at the two times or alternatively the number of surviving bacteria may remain constant during the 10-day storage period.

Discussion

Basic capacity test

In all our experiments the concentrations tested are those recommended by the manufacturers for the specific purposes for which they are used. When the lowest dilution is effective, then it can be assumed that the higher recommended concentrations give at least the same results.

For the phenols, satisfactory results were obtained with substituted phenols at 0.16%, alkyl and aryl phenols at 2% of the commercial product and chloroxylenol at a concentration of 0.245% and 0.14%. Chloroxylenol 0.06% mixed with EDTA was effective against *Pseudomonas aeruginosa.* The other concentrations of the phenols tested gave less interesting results.

In particular, we note an unsatisfactory potency of the solution of alkyl and aryl phenols at 0.5% of the commercial product with the *Staphylococcus aureus* strain in the presence of organic matter. It is interesting that this laboratory experimental finding was confirmed by contamination problems in one hospital where the contaminating strains belonged to the genus *Staphylococcus.* Chlorofen is not effective in most tests at concentrations of 0.008 and 0.04%.

Chlorhexidine at 4% and cetrimide at 0.2% are satisfactory but the solutions of chlorhexidine at 0.05% and the two mixtures of chlorhexidine and cetrimide tested give some problems in particular in the presence of organic material. Contaminations of such disinfectant solutions are discussed by some authors (Lowbury, 1951; Bassett et al., 1970; Bassett et al., 1973).

The mixture of n-alkyl dimethyl benzylammonium chloride and EDTA is satisfactory in the absence of organic material but when serum is present some Gram-negative bacteria especially *Pseudomonas aeruginosa* are more resistant to the action of the product.

Phenylmercuric borate at 0.01% is not satisfactory especially with *Pseudomonas aeruginosa.* Formaldehyde gives unsatisfactory results for the 3 concentrations under tests even the highest concentration of 0.85% is not effective against *Staphylococcus aureus.* At least one aldehyde formulation at 0.147% is satisfactory with the organisms tested in absence of serum but is less potent when serum is added.

All these experiments show that for some concentrations of disinfectants the reduction of the initial number of bacteria does not reach 5 log cycles under our conditions; that is, after a total time of 30 min and with 3 additions of the inoculum.

Resistance test

In the case of a failure of response to our requirements it is interesting to consider the test's ability to grow in the disinfectant solution experiment, also called "resistance" to the contamination. In fact, a product which does not sufficiently reduce the number of bacteria in our basic test is perhaps suitable for long-acting disinfection if this test gives good results and if all the surviving organisms disappear. In contrast, a product which reduces the number of organisms by 5 log cycles but where the surviving bacteria are able to regrow is a product of less interest for disinfection.

It is well known that in some circumstances, in hospitals, micro-organisms are able to proliferate in solutions which are initially bactericidal especially when they are near the limit active concentration. It has been recognized that the end point in a classic disinfection test, of the type suggested, depends not only on the average resistance of the strain but on the most resistant cells in the culture. Such cells can in some cases begin to grow again in the so-called lethal environment. They do not follow the normal logarithmic survivor time curve and the phenomenon can be associated with adaptation (Sykes, 1965). In this respect the most dangerous organism is *Pseudomonas aeruginosa* through its great adaptative capacities by means of inducible enzymes; for this reason we chose it for our test (Adair et al., 1969; Basset et al., 1970, 1973; Bruun and Digranes, 1971; Burdon and Whitby, 1967; Lowbury, 1951). On examination of the results of the trials of "resistance" to the contamination reported in Table 1, one can state: (a) for chloroxylenol and EDTA, the substituted phenols at 0.16% and even formaldehyde at 0.85% gave satisfactory results with *Pseudomonas aeruginosa* in the capacity test; this fact was confirmed by the test of resistance to the contamination in which there was either a reduction or no change in the number of surviving organisms; (b) the unsatisfactory results obtained with chlorofen (0.04%) were confirmed by the re-growth of bacteria after the 10-day storage; (c) on the other hand, unfavourable data recorded in the efficacy trial can be mitigated by those recorded in the test of "resistance" to contamination where the surviving micro-organisms die during the storage. As an example we have chlorhexidine gluconate at 0.05%, the mixtures of chlorhexidine with cetrimide, formaldehyde at 0.085% and 0.51%. This trial could be interesting for the product for which a fast action is not rigorously obligatory; and (d) lastly for the chloroxylenol at concentrations of 0.245 and 0.147%, which were satisfactory in our capacity test, after a conservation period of 10 days, a significant multiplication of the bacteria is recorded.

Conclusions

An attempt has been made to develop a technique for testing disinfectants which takes into account various factors including the choice of the microbial strains, the contact times, the use of hard water and the addition of organic matter. However, we have also borne in mind the necessity of standardization and the need of reproducibility when elaborating the details of the procedure in view of the existing methods, particularly the "new in vitro test" described by Reybrouck (Reybrouck, 1977). In this respect it is certainly possible to further improve the conditions of the test to

achieve some degree of comparability in the area of disinfectant testing (media, inoculum, organic matter,...).

With regard to the interpretation of the results and proposed criteria, one can object that our requirements were too stringent for a laboratory in vitro test. For example, is it a general requirement for a disinfectant to stand repeated additions of bacteria, to be active in the presence of organic matter, in hard water? The practical use of the product is usually restricted to clean conditions. One can accept that it is not necessary for a routine test in quality control, but for a "basis test" it seemed to us that it was better to know exactly the influence of the main factors which determine the efficacy of one product under various practical conditions. It is better to estimate the influence of these factors in a more reproducible in vitro test than in a practical laboratory test. Of course the influence of the main factors, such as organic matter, hard water, detergents and soap, must also be appreciated in such practical tests with regard to the recommended use of the product.

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