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# Bactericidal Activity of Different Generations of Quaternary Ammonium Disinfectants as per EN 1276: A Comparative Study

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**Abstract:** The most challenging issues facing modern medical practices are microbial infections acquired from Hospitals (HAIs). Different generations of disinfectants based on quaternary ammonium compounds (QACs) are used to address the challenges posed by HAIs. However, the antimicrobial efficacy of disinfectants varies significantly because of compositions and factors like hard water and the presence of interfering materials. The utilization of inefficient QACs disinfectants can result in a further increase in the incidents of HAIs and resistance in microbes. In the present study, the impact of organic load and hard water on the antimicrobial efficacy of different generation QACs disinfectants was evaluated using European norm EN 1276.

**Keywords:** Quaternary ammonium compounds, Microbial infections, Interfering substances, Disinfectants, Hospital Acquired infections

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## Introduction

Microbial infections and their fatal effects are the most challenging obstacles faced by modern treatments. These infections have threatened human life in the past and present (Oaks *et al.*, 1992). When acquired from hospitals and healthcare settings, such infections become more deadly (Khan *et al.*, 2017). These infections are commonly referred to as nosocomial infections or hospital acquired infections (HAIs). Situations become worsen when acquired infections are because of the multidrug resistant microorganisms (Dancer, 2014). The overall infection percentage of multidrug resistant organisms was reported as 55.6 % Gram negative, 32.3% Gram positive, and 12.1% yeasts (Cornejo-Juárez *et al.*, 2015).

Multiple factors contribute to HAIs such as patient related factors, hospital related factors and environment related factors. Although it is impossible to eliminate all causes, many may be

controlled, and disinfection and sterilization are the most significant methods of infection management (Jaffar and Paul, 2014). To combat HAIs, chemical disinfectants come in a wide range of classifications. Most widely used are quaternary ammonium compounds (QACs) disinfectants. Based on the synergetic combinations, QACs are categorized as 1st, 2nd, 3rd, 4th and 5th generation QACs (Block, 2001). However, these disinfectants do not yield appropriate results in practice. The inefficiency of QACs disinfectants in actual practice is a matter of deep concern (Gerba, 2015). Literature suggests that interfering substances like organic load reduce the efficacy of the disinfectants differently. Further other factors like temperature and hard water also impact the overall efficacy of chemical disinfectants.

On the other hand, most of the efficacy data available are based on the traditional methods which mostly do not involve the effect of interfering substances (Holah, 1995; Payne *et al.*, 1999). Recent standardized methods like AOAC (Association of Official Analytical Chemists) and EN standards (European Norms) for disinfectant efficacy have been designed in such a way that the impact of the interfering substances can be determined (Sandle, 2017). The impact of interfering substances is not well documented on the distinct generations of QACs. In the present study, the impact of organic load and hard water on the antimicrobial efficacy of different generation QACs disinfectants was evaluated using European norm EN 1276.

## Materials and Methods

*Microbial Culture*:

Bacterial strains of *Escherichia coli* ATCC 10536, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442 and *Enterococcus hirae* ATCC 10541 were obtained from the National Collection of Industrial Microorganisms (NCIM). Cultures were checked for purity and cryopreserved at -20°C in Glycerol broth for further use.

*QACs Disinfectants*:

Nine different generation QACs disinfectants were selected to represent the commonly used disinfectants in the hospitals or healthcare facilities in India. All of the disinfectants were tested at three distinct concentrations (The manufacturer's suggested concentration, a lower concentration, and a higher concentration) and contact time. All QAC disinfectants were suitably coded and further investigation was done using the codes. All tests were carried out for reproducibility and repeatability in duplicate, and the results are presented as averages. All validation controls, including Experimental controls *"A"*, Neutralizer controls "B" and method validation "C" were carried out in parallel with each efficacy test. The QAC disinfectants used in the investigation are listed in Table 1.

*Test suspension "N" and Validation suspension "Nv" Preparation*:

A working suspension of microbial strains was prepared in the diluent. The cell numbers in the test suspension *"N"* were adjusted from $1.5X10^8$ to $5.0X10^8$ cfu/ml. From the test suspension, Validation suspension *"Nv"* was prepared to have $3.0X10^2$ to $1.6X10^3$ cfu/ml. For counting $10^{-6}$ and $10^{-7}$ dilution of test suspension and $10^{-1}$ dilution of validation suspension were plated in duplicate using TSA (Tryptone Soya agar) media. Inoculated plates were placed in an incubation chamber set at 36 ± 1°C for 20 - 24 h.

*Efficacy test "Na"*:

The antibacterial effectiveness of different generations of QACs was investigated by the dilution neutralization method by European standard EN 1276 (BSI, 2010). The QACs disinfectant sample after dilution with hard water (approximately 300 mg/l $CaCO_3$), was added to microbial test suspension *"N"* and an organic load of 0.3% w/v Bovine albumin. The mixture was held at 20 ± 1° C for 5 min. The disinfectant activity was immediately neutralized by mixing 1 ml of the mixture with 8.0 ml of modified Dey-Engley Neutralizing Broth (58.5 g/1000 ml of

Table 1: List of QACs disinfectant samples

| Sample coding | Composition | QACs Generation | Recommended Disinfectant concentration |
|---|---|---|---|
| Sample A | Benzalkonium Chloride (80%): 2.4854 % | First Generation | 0.25% |
| Sample B | Benzalkonium Chloride (80%): 2.4584% | First Generation | 0.25% |
| Sample C | Benzalkonium Chloride (50%) IP : 11.5% | First Generation | 1% |
| Sample G | Alkyl Dimethyl Benzyl Ammonium Chloride 2.37% & Alkyl Dimethyl Ethyl Benzyl Ammonium Chloride 2.37% | Third Generation | 1.5% |
| Sample K | Didecyl Dimethyl Ammonium Chloride: 6.9%w/w | Fourth Generation | 2% |
| Sample P | Didecyl Dimethyl Ammonium Chloride : 7.0% w/w | Fourth Generation | 2% |
| Sample M | Didecyl Dimethyl Ammonium Chloride: 8.74%<br>N-Alkyl Dimethyl Benzyl Ammonium Chloride: 8.19% | Fifth Generation | 0.4% |
| Sample N | Octyl Decyl Dimethyl Ammonium Chloride: 6.510% w/w,<br>Dioctyl Dimethyl Ammonium Chloride: 2.604% w/w<br>Didecyl Dimethyl Ammonium Chloride: 3.906% w/w<br>Alkyl Dimethyl Benzyl Ammonium Chloride: 8.680%w/w | Fifth Generation | 0.4% |
| Sample O | Didecyl Dimethyl Ammonium Chloride: 10.14%w/w<br>N-Alkyl Dimethyl Benzyl Ammonium Chloride: 6.76% w/w | Fifth Generation | 0.4% |

water) and water. Following neutralization, 1.0 ml was cultured in duplicate on TSA culture media and incubated for 20 - 24 h at 36 ± 1°C. Experiment methodology is shown in Figure 1.

*Experimental Control "A"*:

Experimental control was performed to verify that there was no lethal effect of any other parameter in the experiment. To 1.0 ml of interfering material, 1.0 ml validation suspension *"Nv"* was added, and the contents were stirred for 2 min before being held in a water bath regulated at 20° C. After 2 min 8.0 ml of hard water was added and mixed. After the selected contact time, 1.0 ml was plated on TSA culture media and incubated at 36 ± 1°C for 20 - 24 h. The number of survivors was counted and recorded.

*Neutralizer Control "B"*:

To check the toxicity of the neutralizer, 1.0 ml of water was added to the 8.0 ml neutralizer followed by the addition of 1.0 ml validation suspension in a test tube. Contents of the tube were mixed and placed for 5 min ± 10s in a water bath set at 20 ± 1°C. Following the contact period, 1.0 ml from the mixture was plated in duplicate by pour plate or spread plate method using TSA culture media and incubated at 36 ± 1° C. Number of colonies were counted after 20-24 h of growth.

*Method Validation "C"*:

Method validation" C*",* was performed to verify the adequacy of the method and the neutralizer's ability to neutralize the disinfectant carry over

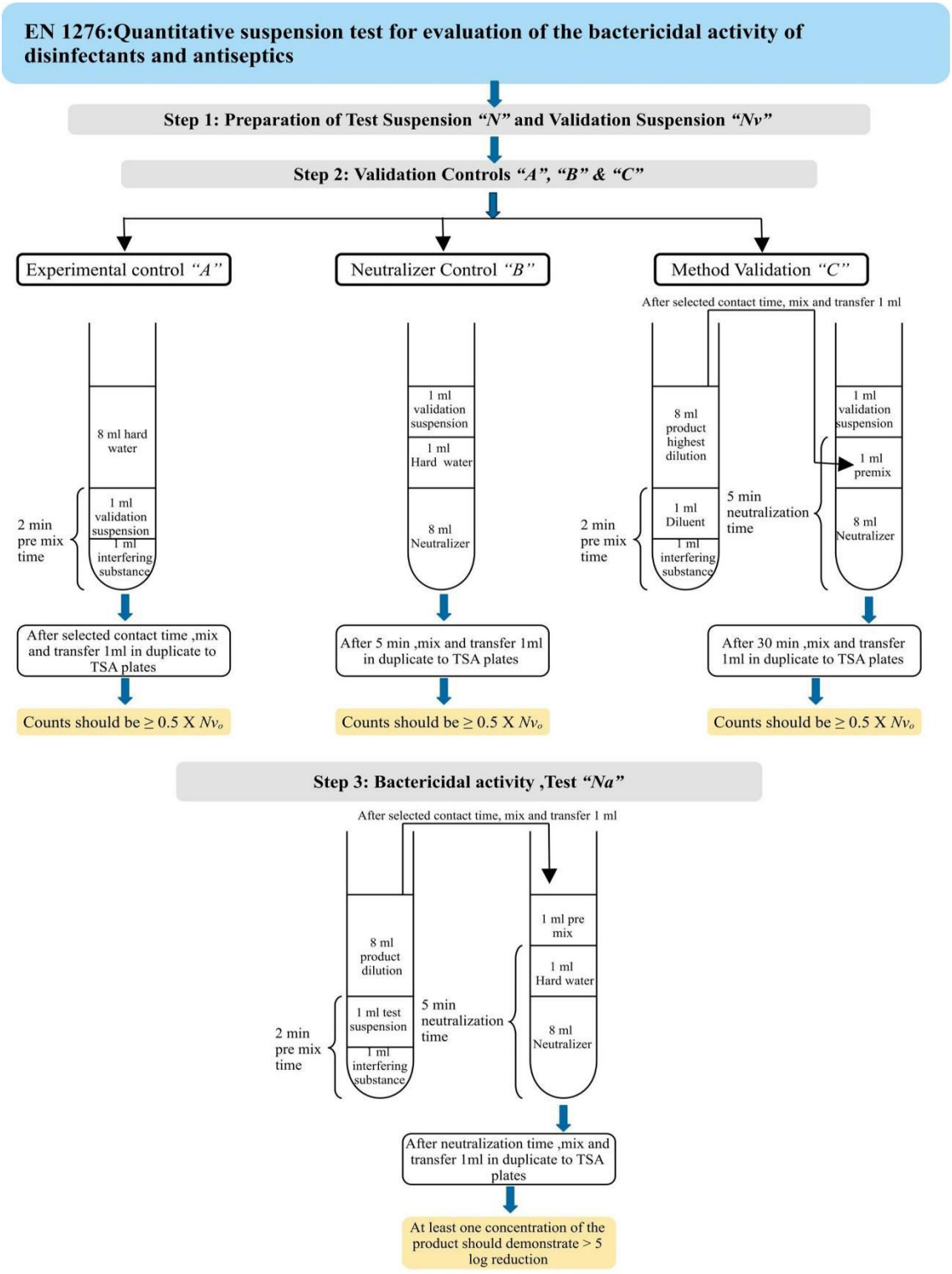


Fig. 1: Methodology flow chart as per EN 1276.

effect. To 1.0 ml of water and diluents, 8.0 ml of the disinfectant's highest concentration was added. The solution was mixed properly and held in a water bath at 20 ± 1°C for desired contact duration. Following the contact time, 1.0 ml mixture was added to 8.0 ml neutralizer. Contents were mixed and kept for a neutralization time of 5 min. Following neutralization, 1.0 ml of validation suspension was added, and the tube was held in a water bath for 30 ± 1 min at 20 ± 1°C. Before the end of the contact period, the contents of the tube were again mixed. At the end of 30 ± 1 min, 1.0 ml was cultured in duplicate by pour plate or spread plate method using TSA culture media and incubated at 36 ± 1° C.

*Log reduction*:

Efficacy of the disinfectant was calculated by deducting the log viable count of survivors in the test *"Na"* from the log number of organisms in the primary inoculums *"$N_0$"* (N X $10^{-1}$).

*Calculations and Acceptance limits as per EN 1276*:

The number of surviving cfu/ml of organisms in the experiments and validation controls was recorded as *Vc* values. Calculations were done as per the standard formulas referred to in EN 1276. When evaluated according to EN 1276, a disinfectant is termed bactericidal if it showed more than five-log reductions.

## Results

Throughout the testing, all validation controls were confirmed to be in accordance with the standard criteria. Bactericidal Efficacy of the QACs based disinfectants varied within the compositions and against the type of organism tested. Out of nine different QACs disinfectants belonging to different generations, only seven passed the test according to the methodology specified in EN 1276. All QACs disinfectants showed >5 Log reduction except sample A (Benzalkonium Chloride 2.4854 %) and sample B (Benzalkonium Chloride 2.4584%) belonging to the first generation. In the case of sample A and sample B, efficacy also varied concerning organism type, where *P. aeruginosa* showed the maximum resistance followed by *E. coli, E. hirae,* and *S. aureus*, respectively in the decreasing order. Detailed results are shown in Table 2.

*Antimicrobial efficacy against P. aeruginosa*:

Both sample A and sample B showed a varied response and failed in the lower concentration (0.1%) at all the contact times. At 0.25% (Manufacturer recommended concentration), both samples A and B failed at 5, 10 min contact times and showed >5 log reduction at 15 min. In the case of higher concentration (0.4%), sample A failed at 5 min while passed at 10 and 15 min of the contact time. On the other hand sample B failed at 5 and 10 min contact time and passed in 15 min showing a log reduction of >5.4124.

*Antimicrobial efficacy against S. aureus*:

In the case of efficacy against *S. aureus*, sample A failed in the lower and the recommended concentration at 5 min contact time while passing at other contact times and concentrations. On the other hand sample B failed to show bactericidal activity against *S. aureus* at 5 min contact times at the lower concentration only.

*Antimicrobial efficacy against E. hirae*:

Against *E. hirae*, sample A showed a <5 log reduction in the lower concentration (0.1%) at 5 min. However, <5 Log reduction was observed in sample B at all contact time. At other concentrations and contact times both A and B showed >5 log reduction.

*Antimicrobial efficacy against E. coli*:

In the case of bactericidal efficacy against *E. coli*, both A and B disinfectants showed < 5 log reduction corresponding to lower concentration at 5, 10, and 15 min contact time. The other two concentrations showed > 5 log reductions at all contact times.

## Discussion

The antimicrobial efficacy of QACs is of utmost importance because of wide-scale use of QACs disinfectants. Over the decades, several methods were developed like simple MIC (Minimum

Table 2: Log reduction of QACs disinfectants at varying concentrations and contact period

| Sample code | Concentration % | contact time (Min) | Log reduction against *P. aeruginosa* | Log reduction against *S. aureus* | Log reduction against *E. hirae* | Log reduction against *E. coli* |
|---|---|---|---|---|---|---|
| A | 0.1 | 5 | **<4.0399** | **<3.9099** | **<3.7903** | **<4.1294** |
| | | 10 | **<4.0399** | >5.2823 | >5.1627 | **<4.1294** |
| | | 15 | **<4.0399** | >5.2823 | >5.1627 | **<4.1294** |
| | 0.25 | 5 | **<4.0399** | **3.9099** | >5.1627 | >5.5017 |
| | | 10 | **4.2774** | >5.2823 | >5.1627 | >5.5017 |
| | | 15 | >5.4123 | >5.2823 | >5.1627 | >5.5017 |
| | 0.4 | 5 | **<4.0399** | >5.2823 | >5.1627 | >5.5017 |
| | | 10 | >5.4123 | >5.2823 | >5.1627 | >5.5017 |
| | | 15 | >5.4123 | >5.2823 | >5.1627 | >5.5017 |
| B | 0.1 | 5 | **<4.0399** | **<3.90991** | **<4.1294** | **<4.1294** |
| | | 10 | **<4.0399** | >5.2823 | **4.1679** | **4.1679** |
| | | 15 | **<4.0399** | >5.2823 | **4.1780** | **4.1780** |
| | 0.25 | 5 | **<4.0399** | >5.2823 | >5.5017 | >5.5017 |
| | | 10 | **<4.2774** | >5.2823 | >5.5017 | >5.5017 |
| | | 15 | >5.4123 | >5.2823 | >5.5017 | >5.5017 |
| | 0.4 | 5 | **<4.0399** | >5.2823 | >5.5017 | >5.5017 |
| | | 10 | **4.6239** | >5.2823 | >5.5017 | >5.5017 |
| | | 15 | >5.4124 | >5.2823 | >5.5017 | >5.5017 |
| C | 0.5 | 5 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 10 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 15 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | 1 | 5 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 10 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 15 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | 2 | 5 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 10 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 15 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| G | 0.75 | 5 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 10 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 15 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | 1.5 | 5 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 10 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 15 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | 2 | 5 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 10 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| | | 15 | >5.4156 | >5.4656 | >5.1685 | >5.5448 |
| K | 1 | 5 | >5.4156 | >5.4469 | >5.1113 | >5.4305 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 10 | >5.4156 | >5.4469 | >5.1113 | >5.4305 |
| | | 15 | >5.4156 | >5.4469 | >5.1113 | >5.4305 |
| | 2 | 5 | >5.4156 | >5.4469 | >5.1113 | >5.4305 |
| | | 10 | >5.4156 | >5.4469 | >5.1113 | >5.4305 |
| | | 15 | >5.4156 | >5.4469 | >5.1113 | >5.4305 |
| | 3 | 5 | >5.4156 | >5.4469 | >5.1113 | >5.4305 |
| | | 10 | >5.4156 | >5.4469 | >5.1113 | >5.4305 |
| | | 15 | >5.4156 | >5.4469 | >5.1113 | >5.4305 |
| P | 1 | 5 | >5.4752 | >5.3747 | >5.1798 | >5.4199 |
| | | 10 | >5.4752 | >5.3747 | >5.1798 | >5.4199 |
| | | 15 | >5.4752 | >5.3747 | >5.1798 | >5.4199 |
| | 2 | 5 | >5.4752 | >5.3747 | >5.1798 | >5.4199 |
| | | 10 | >5.4752 | >5.3747 | >5.1798 | >5.4199 |
| | | 15 | >5.4752 | >5.3747 | >5.1798 | >5.4199 |
| | 3 | 5 | >5.4752 | >5.3747 | >5.1798 | >5.4199 |
| | | 10 | >5.4752 | >5.3747 | >5.1798 | >5.4199 |
| | | 15 | >5.4752 | >5.3747 | >5.1798 | >5.4199 |
| M | 0.2 | 5 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 10 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 15 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | 0.4 | 5 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 10 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 15 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | 0.6 | 5 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 10 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 15 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| N | 0.2 | 5 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 10 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 15 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | 0.4 | 5 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 10 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 15 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | 0.6 | 5 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 10 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| | | 15 | >5.4002 | >5.3663 | >5.1113 | >5.3806 |
| O | 0.2 | 5 | >5.4752 | >5.3401 | >5.1798 | >5.4676 |
| | | 10 | >5.4752 | >5.3401 | >5.1798 | >5.4676 |
| | | 15 | >5.4752 | >5.3401 | >5.1798 | >5.4676 |
| | 0.4 | 5 | >5.4752 | >5.3401 | >5.1798 | >5.4676 |
| | | 10 | >5.4752 | >5.3401 | >5.1798 | >5.4676 |
| | | 15 | >5.4752 | >5.3401 | >5.1798 | >5.4676 |
| | 0.6 | 5 | >5.4752 | >5.3401 | >5.1798 | >5.4676 |
| | | 10 | >5.4752 | >5.3401 | >5.1798 | >5.4676 |
| | | 15 | >5.4752 | >5.3401 | >5.1798 | >5.4676 |

Inhibitory concentration) tests, Suspension tests, carrier tests, and other tests based on mimicking the practical use conditions. These tests are further categorized as qualitative and quantitative (Payne *et al.*, 1999). Most of the efficacy data available are generated by the manufacturers based on the traditional or in-house derived methods. However, the manufacturer's derived methods or traditional methods are mainly based on the concentration and contact time and may not include other factors like organic load, neutralizers and hard water (Sandle, 2020). Hence, using standardized methods always helps to get reliable results. There are mainly two types of standards that are followed worldwide, i.e. EN standards and AOAC methods. In the current study, the antibacterial effect of all selected antimicrobials was determined using the EN1276 standard. EN1276 standard includes bactericidal assessment of disinfectants in exposure to interfering compounds and hardness of the water. Numerous studies have reported that several factors interfere with the efficacy of chemical disinfectants. Such factors include organism type, antimicrobial concentration, exposure period, temperature, pH, hardness of water, and the presence of interfering substances such as organic load (Ridenour and Armbruster, 1948; Tuncan, 1993).

Results of the present study showed that first generation QACs disinfectants (Sample A and Sample B) are not bactericidal in presence of interfering materials and water hardness. However, sample C showed 100% efficacy. This variation can be because of differences in the compositions of first generation QACs samples - A, B and C. Both samples A and B have an active concentration of <3% in comparison to 11% in sample C. Guidelines by CDC (Centers for Disease Control and Prevention) and NCDC (National Centre for Disease Control) suggest that the antimicrobial efficacy of any compound depends on concentration and contact time (Rutala and Weber, 2008; National Centre for Disease Control, 2016). This is well justified in another study that showed that concentration and contact duration have a substantial impact on disinfection effectiveness (West *et al.*, 2018).

Furthermore, disinfection efficacy is affected by the presence of the interfering substances and water hardness. Interfering chemicals such as humic acid and Bovine Serum Albumin (BSA) have been extensively studied, and it has been shown that depending on the type of interfering ingredient, disinfection action is reduced (Gomes *et al.*, 2014). It was also established that the variety of the species, strains, and physiological conditions, as well as disinfection strategies such as molecules, concentrations, and contact duration, impacts disinfectant efficiency. Different isolates of the same species may respond differently to an identical disinfectant, resulting in an effective killing in one example and no impact in others (Buffet *et al.*, 2012).

Hence, regardless of generation type, the presence of these factors renders deadly dosages of QACs disinfectants non-fatal on microorganisms widespread in the hospital environment. In case of QACs it was reported that the presence of two per cent BSA makes up to 80% of the dose ineffective. In case of Human serum and yeast extract, 10 to 100 times the respective dose of QACs is required to achieve disinfection (Lambert and Johnston., 2001).

In another study, QACs were tested against three different microorganisms (*Pseudomonas aeruginosa, Staphylococcus aureus, and Candida albicans*). It was reported that in absence of interfering substances all disinfectants had a similar lethal kill percentage, however, when hard water is introduced, the results demonstrated a strong reduction in efficacy (Araújo *et al.*, 2013).

Thus, using globally defined methodologies that incorporate the interfering material as one key parameter, the influence of the interfering substance on disinfection efficacy should be determined (Lambert and Johnston., 2001). The results showed that higher generation QACs disinfectants are effective in presence of organic load and hard water against *P. aeruginosa, S.*

*aureus, E. hirae, and E. coli*. Such disinfectants can be used in hospitals and healthcare settings for the prevention of HAIs. Furthermore, it will reduce the likelihood of bacteria developing resistance to disinfectants in the healthcare setting. The use of disinfectants merely based on the manufacturer's claims is causing increased incidents of microbes getting resistant to disinfectants (Amini *et al.*, 2020). Hence, the results of the present study indicated that it is very important to access the antimicrobial activity of the intended disinfectant before use and that too with standardized methods.

## Conclusion

The overall results demonstrated that selection of the right disinfectant is very important in the prevention of HAIs. Apart from this, the use of the standardized method for efficacy evaluation is deemed important considering the impact of various interfering substances. The present study indicated that the first generation QACs disinfectants are unable to show the required log reduction in presence of the interfering substance and hard water. Choosing inefficient QACs disinfectants in critical areas of the healthcare facilities will further lead to increased incidents of HAIs. Thus, it emphasizes the careful selection of the disinfectants and methods for antimicrobial efficacy, especially in areas with organic load or other interfering substances.

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