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ORIGINAL ARTICLES

Efficacy Of Combination Of Glutaraldehyde And Benzalkonium Chloride Against Multidrug-Resistant Gram Negative Bacteria Isolated From Hospitals

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ABSTRACT

Due to the growing number of outbreaks of nosocomial infections in hospitals, it has been shown that the appropriate environmental hygienic and disinfection practices can be very helpful to hospital infection control. The aim of this study was to evaluate the efficacy of glutaraldehyde and benzalkonium chloride alone and in combination against antibiotic-resistant hospital Gram-negative bacteria (GNB) isolated. Twenty one isolates of antibiotic-resistance GNB (11 strains of *Escherichia coli*, 5 strains of *Salmonella typhi*, 3 strains of *klebsiella pneumoniae* and 2 strains of *Pseudomonas aeruginosa*) were selected from seventy one GNB which had been collected of some clinical hospitals in Cairo, Egypt. These were evaluated to determine the minimal inhibitory concentration (MIC) values of Glutaraldehyde and Benzalkonium Chloride, separately and in combinations. In addition, to choice one isolate from each strain. MIC for both agents alone or in combination was recorded. Whereas, MIC results of glutaraldehyde was 3000 mg/L for *E.coli, K. pneumoniae, P. aeruginosa and S. typhi*, and MIC results of benzalkonium chloride was 256 mg/L for *K. pneumoniae* and *S. typhi* and also was 64 mg/L and 128 mg/L for *E. coli* and *P. aeruginosa,* respectively. In addition, the microbicidal effect was determined by using quantitative suspension tests $\log_{10}$ reductions of test bacteria after contact times 15 sec., 1 and 5 minutes. The best $\log_{10}$ reductions of glutaraldehyde was concentration 2000 mg/L for *K. pneumonia*e after 1 & 5 minutes, While, it was 3000 mg/L against *E. coli, S. typhi and P. aeruginosa* after 1 & 5 minutes. The $\log_{10}$ reductions of benzalkonium chloride was concentration 256 mg/L for *K. pneumonia*e after one minute, E*. coli* at concentration 512 mg/L after contact 15 second and against*, S. typhi* after 5 minutes at concentration 128 mg/L. and The preferred $\log_{10}$ reductions of combinations was 2000 mg/L of glutaraldehyde plus 8 mg/L of benzalkonium chloride after contact five minutes against *E. coli and S. typhi*, after one minute against *K. pneumonia*e and 15 second contact for *P. aeruginosa*.

Key words:

Introduction

Disinfectants are one of an integral part of good hygienic practice, where they play a fundamental role in infection control practices and help in the avoidance of infections (Rutala and Weber, 1999 and IFH, 2000 &2003).

In spite of, our knowledge of risk factors, prevention and control measures, and the incidence of nosocomial infections has not decreased, and many outbreaks have been caused by new multidrug-resistant pathogens due to excessive use of antibiotics (Bagattini *et al*., 2006; Zarrilli *et al*., 2007). These microorganisms are resistant to the majority of antibiotics and to many disinfectants, which has resulted in an increase in environmental contamination (Levy, 2000 and Webster *et al*., 2000).

In many cases, it has been demonstrated that the molecular mechanisms responsible for antibiotic resistance are the same as those implicated in lack of susceptibility to biocides (Chuanchuen *et al*., 2001; Heir *et al*., 2001; Sidhu *et al*., 2002; Fluit and Schmitz, 2004 and Piddock, 2006). Biocides have a broader spectrum of activity than antibiotics, while antibiotics tend to have specific intracellular targets, biocides may have multiple targets. So, disinfectants, unlike antibiotics, have a broad spectrum of antimicrobial activity and generally act on several targets in microbial cells (Russell, 2003).

In general, gram negative bacteria are intrinsically more resistant to disinfectants than gram positive bacteria, mainly because of their relatively impermeable outer membrane (McDonnell and Russell, 1999), such as Benzalkonium chloride (one of QACs) was more effective on Gram positive than Gram negative bacteria (Fazlara and Ekhtelat, 2012).

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Quaternary ammonium compounds (QACs) are cationic biocides that are commonly used as disinfectants. Benzalkonium chloride (BAC), a synthetic antimicrobial agent with a broad spectrum antimicrobial (Holah *et al*., 2002; Carson *et al*., 2008; and Walton *et al*., 2008), is a QAC that is widely used as disinfectant and cationic surface active agent for sanitation in food processing lines and surfaces in the food industry (Kuda *et al*., 2008), as clinical disinfectant and antiseptic (topical) in health care facilities and domestic households such and as antimicrobial preservative in drugs in low concentration (Pernak *et al*., 1999; Mangalappalli-Illathu and Korber, 2006). BAC is the product of a nucleophilic substitution reaction of alkyldimethylamine with benzyl chloride (Pernak *et al*., 1999).

Glutaraldehyde is an important dialdehyde that has found usage as disinfectant and sterilant, where glutaraldehyde has a broad spectrum of activity against bacteria and their spores, fungi and viruses. Gorman *et al*. (1980), Beauchamp *et al*. (1992) and McDonnell and Russell (1999) demonstrated that glutaraldehyde possessed high antimicrobial activity and its broad biocidal activity arises from the propensity to cross-link with NH2 groups on protein chains, such as lysine residues in microbial cell walls.

Combinations of antimicrobials, whether they are antibiotics, disinfectants or preservatives, which demonstrate a synergistic effect, are much sought after. In the clinical area, combination antibiotic therapy is used in an attempt to avoid the emergence of resistance, it can broaden the bacterial spectral range, and synergy between the antimicrobials can lead to a better clinical outcome (Ryan *et al*., 1981; Paull and Marks, 1987; Owens *et al*., 1997 and Acar, 2000).

Currently in Europe, the biocidal Products Directive has almost halted any new biocide discovery research. So many biocide producers and suppliers allow product development without the extreme cost associated with new compounds through combinations of known and registered biocides. One very real justification for the development of synergistic antimicrobial combinations is that they are patentable. The expense of developing a new antimicrobial vs. the cost of finding a combination of known and already registered compounds is an easy justification for research into synergy. Furthermore, when a synergistic combination is found, the patent ensures the possible financial return on the investment (Lambert *et al*., 2003).

For the study of combinations of disinfectants, a simple experimental approach to the problem was to compare kinetic data of individual compounds with mixtures of the same compounds. An elevated level of microbial inactivation of the mixture relative to the additive effect of the individual components was all that was required to show the existence of synergy (Hugbo, 1976).

The aim of this study was to evaluate the efficacy of glutaraldehyde and benzalkonium chloride alone and in combination against multidrug-resistant gram negative bacteria isolated from clinical hospitals.

Materials And Methods

Clinical isolates of gram negative bacteria:

Twenty one isolates of antibiotic-resistance GNB (*Escherichia coli, Salmonella typhi, klebsiella pneumoniae* and *Pseudomonas aeruginosa)* were selected from seventy one GNB which had been collected of some clinical hospitals in Cairo, Egypt (Ain-Shams University Hospitals; Abu El-Reish Hospital and Al-Borg private Hospital). The collected isolates were identified by using selective media and biochemical tests.

Media used:

Media used for cultivation, purification and identification were Nutrient broth (Lab, UK), Nutrient agar (Lab, UK), Mueller–Hinton agar media (LAB, UK), Mueller–Hinton broth media (LAB, UK), MacConkey agar (Oxoid, UK), EMB agar (Oxoid, UK), Brilliant green agar (Britania, Argentina), Bismuth sulfite agar (Titan biotech), Selenite cystiene broth (Oxoid, UK), SS agar (Oxoid, UK), Pseudomonas agar F & B (Difco, USA), Tryptic Soy Agar (Britania, Argentina) and Tryptic soy broth media (Britania, Argentina) and biochemical tests for identification were Methyl red test and Voges-Proskauer test, Citrate test, Urease Test, Oxidase Test (Himedia), Lactose Test, Lysine Iron Agar and Motility Indole Ornithine Medium.

Antibiotic discs: (purchased from Oxoid, UK):

Antibiotics selected for testing recommended by Clinical Laboratory Standards Institute (CLSI) (USA), document M100-S18. antibiotic discs used for *enterobateriaceae* were Ampicillin – AM (10 µg), Amoxicillin-clavulanic acid – AMC (20/10µg), Gentamicin – GN (10 µg), Piperacillin-tazobactam PIP/TAZ (100/10 µg), Amikacin – AM (30 µg), Ciprofloxacin – CIP (5 µg), Cefotaxime - CTX (30 µg), Cefuroxime – CXM (30 µg), Norfloxacin – NOR (10 µg), Cefoxitin – FOX (30 µg), Imipenem – IPM (10 µg) and antibiotic discs used for *Pseudomonas aeruginosa* were Gentamicin – GN (10 µg), Piperacillin-tazobactam PIP/TAZ (100/10 µg),

Amikacin – AK (30 µg), Ciprofloxacin – CIP (5 µg), Cefotaxime - CTX (30 µg), Norfloxacin – NOR (10 µg), Imipenem – IPM (10 µg), Ceftazidime - CAZ (30 µg) and Piperacillin – PRL(100 µg).

Disinfectants:

Disinfectants used were glutaraldehyde (Cidex 2.5%) and benzalkonium chloride (10%).

Neutralizing agents:

Neutralizing agents used for glutaraldehyde were Glycine 1% + tween 80 0.5% and for benzalkonium chloride were lecithin 0.7% + tween 80 0.5 %. (Gardner and Peel, 1986).

Antibiotics susceptibility testing (Disk diffusion method):

The susceptibility of the seventy one bacterial isolates to antibiotics was determined by the Kirby-Bauer disk diffusion method (Bauer *et al.,* 1966). Then the more susceptible and the multidrug-resistance GNB (*Escherichia coli, Salmonella typhi, Klebsiella pneumoniae* and *Pseudomonas aeruginosa)* were selected for further testing to compare between the two groups in their susceptibility. Also the relation between resistance to antibiotics and disinfectants has been investigated.

Performing the Disk Diffusion Test:

Briefly, antimicrobial disks were applied to the surface of a Mueller-Hinton agar plate previously inoculated by identified bacterial isolates. The plates were incubated at 35 ± 2°C for 16 to 18 hours, each plate examined and the diameters of the zones of complete inhibition measured, including the diameter of the disk and the results were interpreted as susceptible, intermediate, or resistant to the agents according to CLSI M100-S18 (2008).

Determination of minimal inhibitory concentration (MIC) of biocides:

MICs of disinfectants were determined by the agar dilution method, according to the protocol recommended by the CLSI (formerly NCCLS) in document M100-S14 (2004). Briefly, appropriate dilutions of antimicrobial or disinfectant solutions were added to Mueller–Hinton agar that had been allowed to equilibrate in a water bath to 50–55°C. The agar and disinfectant solution were mixed thoroughly, and the mixture was poured into Petri dishes on a level surface to result in an agar depth of 3–4 mm. Each bacterial culture was adjusted to a turbidity equivalent to that of a 0.5 McFarland standard (~1–9×10^8 cfu/mL for most species) and was then diluted 1:10 in sterile Mueller–Hinton broth. A 5 µL aliquot of each diluted bacterial suspension containing ~10^4 cfu was spotted onto the agar surface using an automatic pipette within 15 min of preparation and the plates were incubated at 35°C for 20 hrs. The MIC was recorded as the lowest concentration of the disinfectant that completely inhibited growth (Kawamura-Sato *et al*., 2010). Then isolates with reduced susceptibility to disinfectants was selected for further investigation.

Combination of disinfectants:

Checkerboard assay was used to evaluate the antimicrobial efficacy of glutaraldehyde and benzalkonium chloride upon combination against selected isolates with reduced susceptibility to disinfectant.

The concentrations tested for each disinfectant were lower and higher than its MIC concentration. Organisms and agar dilution plates were prepared as for the MIC determination. To evaluate the effect of the combinations, the fractional inhibitory concentration (FIC) index was calculated using the formulae (Satish *et al.,* 2005)

$$\text{FIC index} = \text{FIC}_X + \text{FIC}_Y = \frac{(X)}{(\text{MIC}_X)} + \frac{(Y)}{(\text{MIC}_Y)}$$

Where the X, Y is the disinfectants being tested, (MICx and MICy) are the MICs obtained for each disinfectant when tested alone, and ((X) and (Y)) are the concentrations of each disinfectant at the lowest effective combination.

Result were interpreted as, Synergy (FIC index <0.5) where there was a fourfold or greater decrease in MIC of both disinfectants in combination compared with the disinfectants tested individually, Partial synergy where there was a four-fold or greater decrease in MIC with one agent and with a two-fold decrease in the other agent

(FIC >0.5 but <1), Additive where there was a two-fold drop in MIC with both agents (FIC = 1), Indifference where there was no change in MIC whether the agents were tested alone or in combination (FIC >1 but <4) or Antagonistic when there was a four-fold increase in MIC for both agents when the disinfectants were tested in combination as compared with results when each disinfectant was tested alone (FIC≥4) (Satish *et al.,* 2005).

Evaluation of bactericidal activity: suspension test:

One ml of the tested bacterial isolate suspension was added to 9 ml of the disinfectant solution for each prepared concentration. Then, after a predetermined exposure time (15 sec., 1 and 5 min.), one ml of the disinfectant-organism mixture was added to 9 ml of specific neutralizer. After a neutralization time of 5 min, further dilutions were made in diluents (trypticase soy broth) and 1ml from each dilution was inoculated onto plates of trypticase soy agar. Plates were incubated at 37°C for 24 h. Control test carried out with the same method except 9 ml distilled water used instead of disinfectant.

After incubation the number of surviving bacterial isolates were counted and compared with the initial inoculums size. The decimal-log reduction rate, microbicidal effect (ME) can be calculated, using the formula ME = log *N*c - log *N*D (*N*c being the number of colony-forming units developed in the control series in which the disinfectant is replaced by distilled water, and *N*D being the number of colony-forming units counted after exposure to the disinfectant) according to CEN Standards (European Committee for Standardization, 1997). The pour-plate technique as well as surface plates may also be used for sub-culturing (Reybrouck, 1998).

Results and Discussion

Antibiotics susceptibility:

Table 1: Antibiotics susceptibility for GNB isolates from clinical hospitals.

Numbers of the isolates.	Identified resistant isolates	Antibiotics susceptibility
4	*E. coli*	AM, CIP, NOR
5		AM, CTX, CXM
68		CIP, CXM, NOR
99		AM, CTX, CXM
17		AM, AMC, CN, TZP, AK, CIP, CTX, CXM, NOR, FOX
27		
29		
31		
32		
33		
35		
28	*K pneumoniae*	AM, AMC, CN
70		AM, AMC, TZP, CXM
37		AM, AMC, CN, TZP, CIP, CTX, CXM, NOR, FOX, IPM
49	*P. aeruginosa*	CTX, CAZ, PRL
48		CN, TZP, AK, CIP, CTX, NOR, CAZ, PRL
72	*S. typhi*	AM, CTX, CXM
88		
93		AM, AMC, CN, CXM, FOX, IPM
91		AM, AMC, CN, TZP, CIP, CTX, CXM, NOR
92		AM, AMC, TZP, AK, CTX, CXM, NOR, FOX

AM: Ampicillin, AMC: Amoxicillin-clavulanic acid, GN: Gentamicin, PIP/TAZ: Piperacillin-tazobactam, AK: Amikacin, CIP: Ciprofloxacin, CTX: Cefotaxime, NOR: Norfloxacin, CXM: Cefuroxime, FOX: Cefoxitin, IPM: Imipenem, CAZ: Ceftazidime, PRL: Piperacillin.

The results of table (1) show the susceptibility of the twenty-one Gram-negative bacterial isolates (11 strains of *Escherichia coli,* 3 strains of *Klebsiella pneumoniae*, 5 strains of *Salmonella typhi* and 2 strains of *Pseudomonas aeruginosa*) to antibiotics. Seven strains of *Escherichia coli* were resisting to ten and four strains to three antibiotics, while, one strain of *K. pneumoniae* was resisting to ten, one strain to four and one strain to three antibiotics. But, two strains of *S. typhi* were resisting to eight, one resist to sex and one resist to three antibiotics, and one strain of *P. aeruginosa* was resisting to eight and one resist to three antibiotics.

Susceptibility to disinfectants:

The MIC of glutaraldehyde and benzalkonium chloride for twenty one clinical isolates are shown in table (2). We noticed that the MIC of disinfectants for multidrug resistant isolates (*E. coli, K. pneumonia*e and *S. typhi*) was higher than the MIC of disinfectants recorded for antibiotic-sensitive isolates, except *P.aeruginosa* the MIC were the same concentrations.

Table 2: Minimal inhibitory concentration (MIC) values of glutaraldehyde and benzalkonium chloride.

Identified resistant isolates	R*	Numbers of the isolates.	Minimal inhibitory concentrations (MIC)			
			Glutaraldehyde		Benzalkonium Chloride	
			mg/L	%	mg/L	%
E. coli	R^3	4	3000	0.3	32	0.0032
		5	2000	0.2	32	0.0032
		68	2000	0.2	32	0.0032
		99	2000	0.2	32	0.0032
	R^{10}	17	2000	0.2	64	0.0064
		27	3000	0.3	64	0.0064
		29	3000	0.3	64	0.0064
		*31	3000	0.3	64	0.0064
		32	3000	0.3	64	0.0064
		33	3000	0.3	64	0.0064
		35	3000	0.3	64	0.0064
K. pneumoniae	R^3	28	2000	0.2	128	0.0128
	R^4	70	2000	0.2	128	0.0128
	R^{10}	37	3000	0.3	256	0.0256
P. aeruginosa	R^3	49	3000	0.3	128	0.0128
	R^8	48	3000	0.3	128	0.0128
S. typhi	R^3	72	2000	0.2	128	0.0128
		88	2000	0.2	128	0.0128
	R^6	93	3000	0.3	256	0.0256
	R^8	*91	3000	0.3	256	0.0256
		92	2000	0.2	256	0.0256

R*. Resistant phenotype to numbers of antibiotics.

* Bacteria isolate numbers 31 and 91 were selected randomly.

The results of table (2) show that MIC for glutaraldehyde was 0.3% (3000 mg/L) for *E. coli, K. pneumoniae, P. aeruginosa and S. typhi*, which showed similarity to the results of Priscila (2009) with MIC results were 2750-3750 mg/L for *E. coli.* The determined MIC of benzalkonium chloride was 0.0256% (256 mg/L) for *K. pneumoniae* and *S. typhi* and also was 0.0064% (64 mg/L) and 0.0128% (128 mg/L) for *E. coli* and *P. aeruginosa,* respectively. This results were nearest to the results obtained by Priscila (2009) which stated that MIC values of QACs were 117 and 156 mg/L for gram negative bacteria, and similar to the MIC values for vegetative cells of *E. coli* (MIC between 59-78 mg/L).

Four bacterial isolates, *Escherichia coli* (isolate number 31), *Klebsiella pneumoniae* (isolate number 37), *Salmonella typhi* (isolate number 91) and *Pseudomonas aeruginosa* (isolate number 48), were selected for further investigation according to their reduced susceptibility to disinfectants and the lowest MIC of disinfected than selected concentrations had no effect.

Combination of disinfectants:

By using checkerboard assay to evaluate the antimicrobial efficacy of glutaraldehyde and benzalkonium chloride upon combination was shown in table (3).

Table 3: The antimicrobial efficacy of combination between glutaraldehyde and benzalkonium chloride.

Concentrations of disinfectant				*E. coli*	*K. pneumoniae*	*P. aeruginosae*	*S. typhi*
Glutaraldehyde		Benzalkonium chloride					
mg/L	%	mg/L	%				
3000	0.3%	512	0.0512%	-ve	-ve	-ve	-ve
		256	0.0256%	-ve	-ve	-ve	-ve
		128	0.0128%	-ve	-ve	-ve	-ve
		64	0.0064%	-ve	-ve	-ve	-ve
		32	0.0032%	-ve	-ve	-ve	-ve
2000	0.2%	512	0.0512%	-ve	-ve	-ve	-ve
		256	0.0256%	-ve	-ve	-ve	-ve
		128	0.0128%	-ve	-ve	-ve	-ve
		64	0.0064%	-ve	-ve	-ve	-ve
		32	0.0032%	-ve	-ve	-ve	+ve
1000	0.1%	512	0.0512%	-ve	-ve	-ve	-ve
		256	0.0256%	-ve	-ve	-ve	-ve
		128	0.0128%	-ve	+ve	-ve	+ve
		64	0.0064%	-ve	+ve	+ve	+ve
		32	0.0032%	+ve	+ve	+ve	+ve

-ve = no growth +ve = growth

The data presented in table (3) recorded that the concentration 2000 mg/L of glutaraldehyde plus 64 mg/L of benzalkonium chloride is the lowest concentration which inhibits the growth of all bacterial isolates. Where, MIC for both agents when the biocides were tested in combination as compared with results when each biocide was tested alone, they shown partial synergy (Satish *et al.,* 2005). The FIC index values (table 4) stated a partial synergistic antimicrobial activity of glutaraldehyde-benzalkonium chloride against all tested bacterial isolates (FIC >0.5 but <1).

Table 4: The fractional inhibitory concentration index (FIC index) of glutaraldehyde and benzalkonium chloride.

Identified resistant isolated	Type of biocide	MIC of biocide		FIC of each biocide	FIC index	Outcome
		Alone	In combination			
E.coli	Glu	3000	2000	0.66	0.91	Partial synergy
	BAC	64	8	0.25		
K. pneumoniae	Glu	3000	2000	0.66	0.785	Partial synergy
	BAC	256	32	0.125		
P. aeruginosa	Glu	3000	2000	0.66	0.91	Partial synergy
	BAC	128	32	0.25		
S. typhi	Glu	3000	2000	0.66	0.91	Partial synergy
	BAC	256	64	0.25		

Glu = Glutaraldehyde BAC = Benzalkonium choride

Efficacy of disinfectants:

Quantitative suspension tests are presented as $\log_{10}$ reductions of test bacteria after exposure times 15 sec., 1 and 5 min. The data are presented in tables 5 & 6 for each disinfectant separately but the data in table (7) for combinations of glutaraldehyde with benzalkonium chloride.

The results of the tests were interpreted in accordance with CEN Standards (European Committee for Standardization, 1997). According to Standards, an efficacious biocide must reduce the initial count by 4 or 5 log units, and the efficacy is estimated by the ratio between the number of microorganisms in the starting solution (inocula of 10^8 CFU/ml bacteria) and number of colonies surviving on neutralization plates.

In Gram negative bacteria, resistance mechanisms are more complicated since these organisms possess an inner and an outer membrane. The latter membrane has a clear role in modulating the accessibility of a cell to preservatives and other small molecules; the lipopolysaccharide layer is of crucial importance in this respect (Helander *et al*., 1997 and Brula and Cooteb, 1999).

Table 5: Microbicidal effect of glutaraldehyde as determined by the quantitative suspension test after exposure time 15 sec, 1 and 5 min.

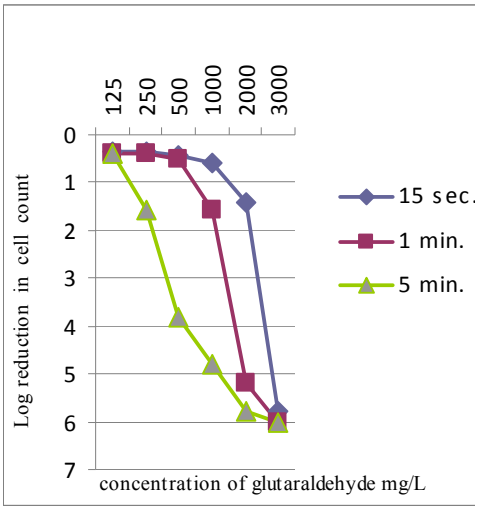
Identified resistant isolates	Microbicidal effect (ME) at concentrations of glutaraldehyde																	
	125 mg/L			250 mg/L			500 mg/L			1000 mg/L			2000 mg/L			3000 mg/L		
	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.
E. coli	0.35	0.38	0.4	0.36	0.4	1.56	0.43	0.5	3.8	0.6	1.59	4.8	1.4	5.2	5.8	3.96	6	6
K. pneumoniae	0.26	1.04	2.8	1.03	1.36	3.4	1.18	1.48	4.4	3.14	6	6	4.6	6	6	6	6	6
P. aeruginosa	0.01	0.04	0.09	0.04	0.12	1.14	0.09	0.24	3.98	0.12	1.16	6	1	4.78	6	3.5	6	6
S. typhi	0.72	0.83	1.1	0.75	0.84	1.89	0.87	2.7	2.8	1.24	2.79	3.08	2.9	4.35	4.5	3.3	6	6

The obtained results in table (5) and figure (1) showed that the germicidal effect for glutaraldehyde was hundred percent ($\log_{10}$) reduction against tested bacteria ***K.pneumoniae*** at concentration 1000 mg/L (0.1%), 2000 mg/L (0.2%) after exposure time 1 and 5 min. and 3000 mg/L (0.3%) after 15 sec., 1 & 5 min. While, against ***P.aeruginosa*** was at concentration1000 mg/L (0.1%), 2000 mg/L (0.2%) after exposure time 5 min and 3000 mg/L (0.3%) after 1 & 5 min. Also, the hundred percent ($\log_{10}$) reduction against ***E.coli and S. typhi*** was at concentration 3000 mg/L (0.3%) after 1 & 5 minutes.

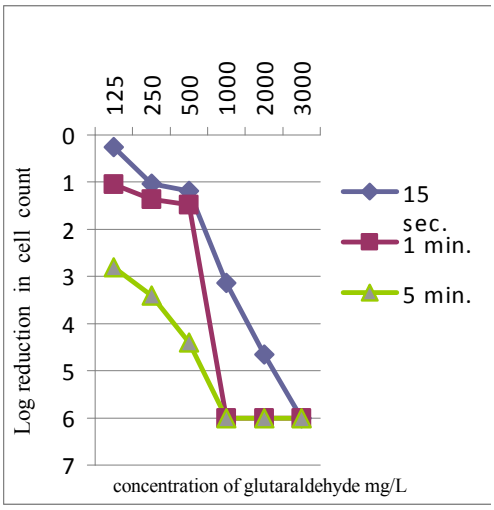
These results are attributed to the mechanism of action of glutaraldehyde involves a strong association with the outer layers of bacterial cells, inhibition of RNA, DNA, and protein synthesis (McGucken and Woodside, 1973), prevention of sodium lauryl sulfate-induced lysis in *E. coli* (Munton and Russell, 1973), these bactericidal studies demonstrated (Power, 1995) a strong binding of glutaraldehyde to outer layers of organisms such as *E. coli* (Hughes and Thurman, 1970; Munton and Russell, 1970; Munton and Russell, 1971; Munton and Russell, 1972; Munton and Russell, 1973 and Gorman and Scott, 1977).

Table 6: Microbicidal effect of benzalkonium chloride as determined by the quantitative suspension test after exposure time 15 sec, 1 and 5 min.

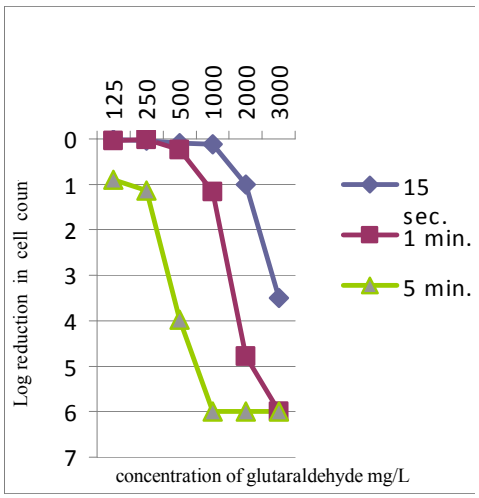
Identified resistant isolates	Microbicidal effect (ME) at concentrations of benzalkonium chloride																	
	16 mg/L			32 mg/L			64 mg/L			128 mg/L			256 mg/L			512 mg/L		
	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.
E. coli	0.07	1.33	2.37	2.04	2.34	3.06	2.28	3	3.24	3.04	3.4	3.45	3.4	3.7	3.97	6	6	6
K. pneumoniae	0.09	0.12	2.3	0.02	0.23	3.5	1.1	3.2	3.68	1.12	3.9	6	1.9	6	6	3.3	6	6
P. aeruginosa	0.05	0.23	1.33	0.07	1	3.46	1.04	2.38	4	3.02	3.46	4.29	4	4.26	6	6	6	6
S. typhi	0.06	0.83	1.87	0.9	1.84	3.23	2.15	2.3	4.23	2.76	3.23	6	6	6	6	6	6	6



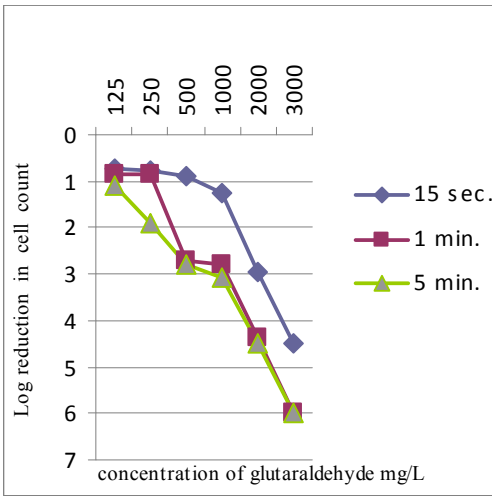
(a) *E. coli*,



(b) *K. pneumoniae*



(c)) *P. aeruginosa*



(d) *S. typhi*

Fig. 1: Microbicidal effect of glutaraldehyde as determined by the quantitative suspension test after exposure time 15 sec, 1 and 5 min.

The results in table (6) and figure (2) show that *E. coli* exhibited hundred percent reduction in concentration 512 mg/L (0.512%) after 15 sec., 1 & 5 minutes. While, *P. aeruginosa* was killed at concentration 512 mg/L (0.512%) after 15 sec., 1 & 5 minutes and at concentration 256 mg/L (0.256%) after exposure time 5 minutes. But, *K. pneumoniae* was hundred percent reductions at concentration 256 mg/L (0.256%) and 512 mg/L (0.512%) after one & five minutes for two concentrations, also, was destroyed completely in concentration 128 mg/L (0.128%) after 5 minutes only. And at concentrations 128 mg/L (0.128%), 256 mg/L (0.256%) and 512 mg/L (0.512%) *Salmonella typhi* was the hundred percent reductions after 5 minutes.

These results were in agreement with that of Bridier *et al*. (2011), they reported that *E. coli* and *P. aeruginosa* were high susceptible to benzalkonium chloride in all concentrations and *E. coli* did not demonstrate significant resistance variability to the benzalkonium chloride. Bruinsma *et al*. (2006) suggest that for *P. aeruginosa*, the electrostatic interactions between the negatively charged cell surface and the positively charged BAC play a key role in its antimicrobial efficiency. One explanation for the heterogeneity in QAC against

Pseudomonas could be explained by its ability to adapt to biocides, notably to quaternary ammonium compounds (Méchin, 1999 and Johnson, 2002). Also, the results were more or less similar to the results obtained by Fazlara and Ekhtelat (2012), they showed that BAC in 160 mg/L concentration was able enough to kill all target bacteria (*Salmonella typhimurium, Escherichia coli* and *Pseudomonas aeruginosa*) during 24 h contact time and proved that *Escherichia coli* to be the second sensitive microorganisms with MIC and MBC equal to 40 and 45 mg/L respectively. The results obtained were not similar with that obtained by Guimarães *et al*. (2000) who found that antibiotic resistant hospital strains of. *K. pneumoniae* and *E. coli* strains were resistant to the QAC.

Table 7: Microbicidal effect of combination between glutaraldehyde and benzalkonium chloride as determined by the quantitative suspension test after exposure time 15 sec, 1 and 5 mins.

Identified resistant isolates	2000mg/L glutaraldehyde plus 8 mg/L benzalkonium chloride			2000mg/L glutaraldehyde plus 16 mg/L benzalkonium chloride			2000 mg/L glutaraldehyde plus 32 mg/L benzalkonium chloride			2000mg/L glutaraldehyde plus 64 mg/L benzalkonium chloride		
	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.	15 sec.	1 min.	5 min.
E. coli	0.02	0.01	6	0.07	6	6	6	6	6	6	6	6
K. pneumoniae	0.1	6	6	6	6	6	6	6	6	6	6	6
P. aeruginosa	6	6	6	6	6	6	6	6	6	6	6	6
S. typhi	0.72	5.95	6	3.05	6	6	3.3	6	6	3.84	6	6

The recorded results in table (7) and figure (3) clarified the decimal $\log_{10}$ reduction of combination between glutaraldehyde at concentration 2000mg/L and benzalkonium chloride at four concentrations, 8, 16, 32 and 64 mg/L after 15 second, one and five minutes exposure time.

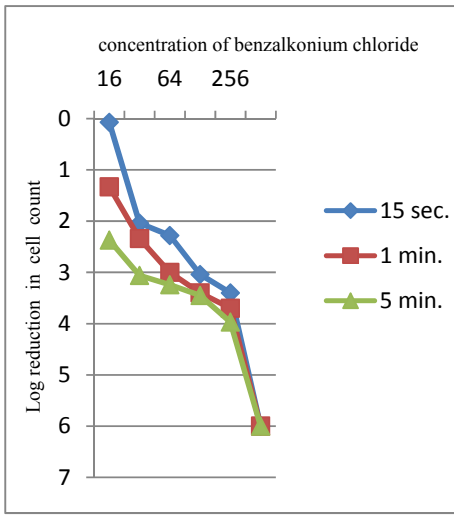
The data shown that *P. aeruginosa* was highly susceptible to all concentrations of the mixture (2000 mg/L glu plus 8, 16, 32 or 64 mg/L BAC) where germicidal effect was hundred percent after 15 second expoure time.

Also, *K. pneumoniae* was similar to *P. aeruginosa* in susceptibility where microbicidal effect was hundred percent after one and five minutes contact at concentration equal 2000mg/L of glu. with 8 mg/L of BAC and in all remaining concentrations after 15 seconds contact time.

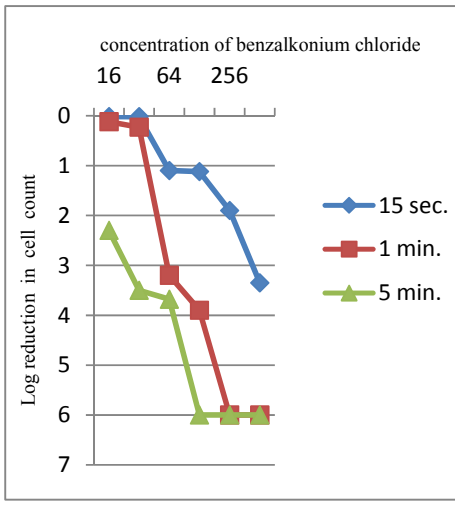
On the other hand, *E. coli* was more resistant than *K. pneumoniae* and log reduction was hundred percent after five minutes at concentration (2000mg/L glu plus BAC 8mg/L) then follow in the second concentration (2000mg/L glu plus 16mg/L BAC) after one and five minutes contact, and the remaining concentrations (2000mg/L glu plus 32 or 64 mg/L BAC) started in 15 seconds contact time.

But, *S. typhi* was more resistant than *E. coli*, *K. pneumoniae* and *P. aeruginosa* , where was completely killed after five minutes contact at concentration (2000mg/L glu plus 8mg/L BAC) and after one minutes exposure times at remaining concentrations(2000mg/L glu plus 16, 32 or 64 mg/L BAC).

The results above were similar to that reported by Soliman *et al*. (2009) who said that quaternary ammoniumgluteraldhyde combination (TH4®) although they are not proven to be environmentally safe; they are the most powerful disinfectants because of the synergistic action of the quaternary ammonium and gluteraldhyde bases. Where TH4® starting to show high efficacy against *Pseudomonas aureuginosa* after 5 min ($p<0.0001$), achieved 100% efficacy against *Escherichia coli* after 5 min ($p<0.0001$) and after 5 min ($p<0.0001$) with killing efficacy (99.99%) and achieved the 100% efficacy after 10 min ($p<0.0001$).



(a) *E. coli*



(b) *K. pneumoniae*

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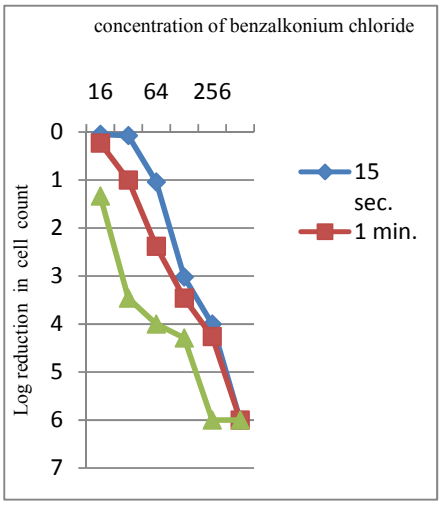
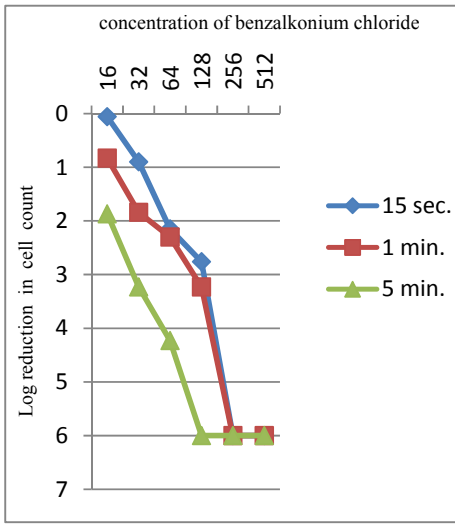
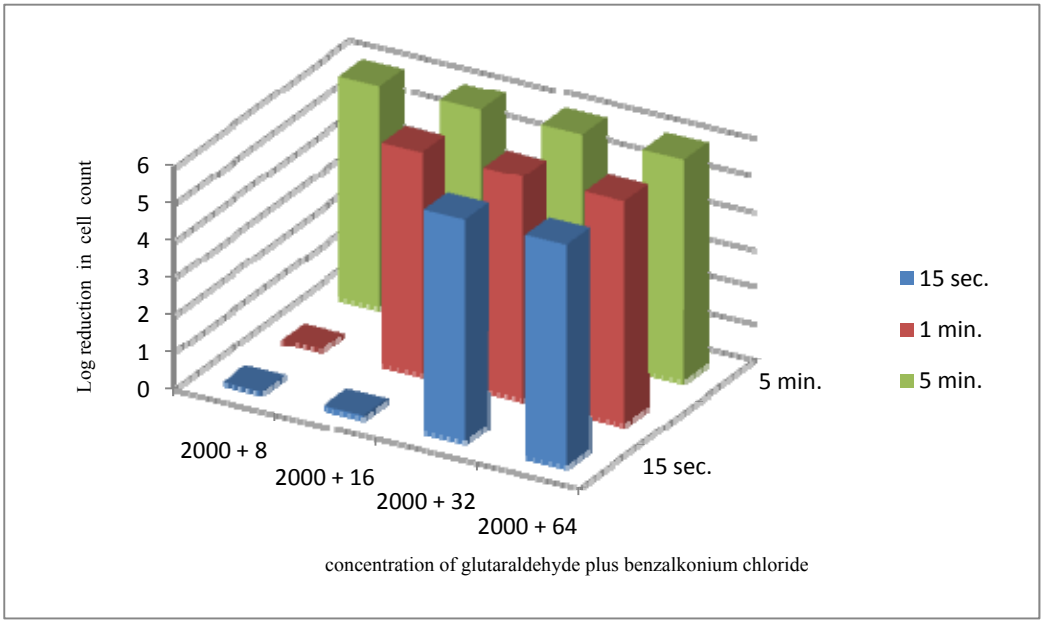




(c)) *P. aeruginosa* (d) *S. typhi*

Fig. 2: Microbicidal effect of benzalkonium chloride as determined by the quantitative suspension test after exposure time 15 sec, 1 and 5 min.

The recording data in table (7) represented the effect of disinfectant combination as well as the existence of synergy, a very high degree of synergy was observed, these results was in agreement with Hugbo (1976); Denyer *et al*. (1985) and Denyer (1990) they stated the antimicrobial synergy essentially uses a single axiom as a base – that a synergy may occur if antimicrobials with different target sites are blended together.



(a) *E.coli*,

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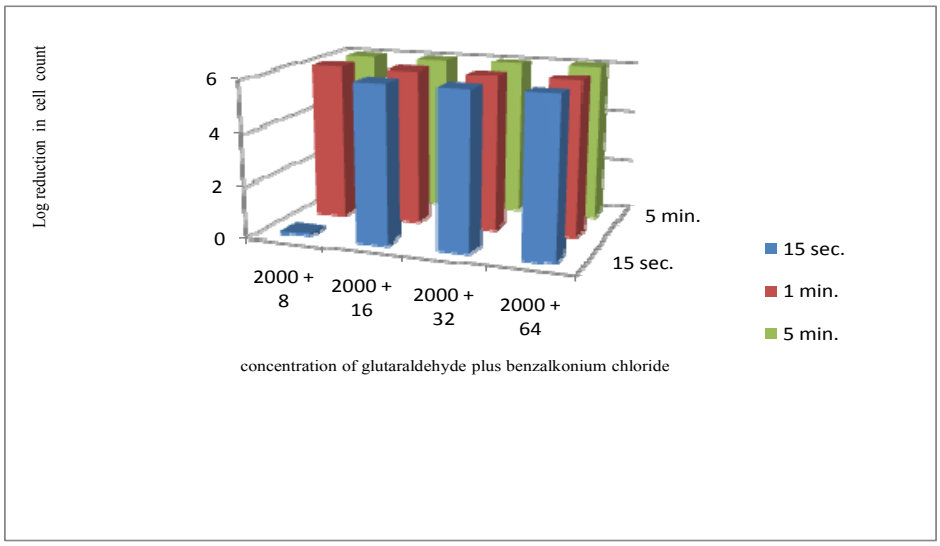
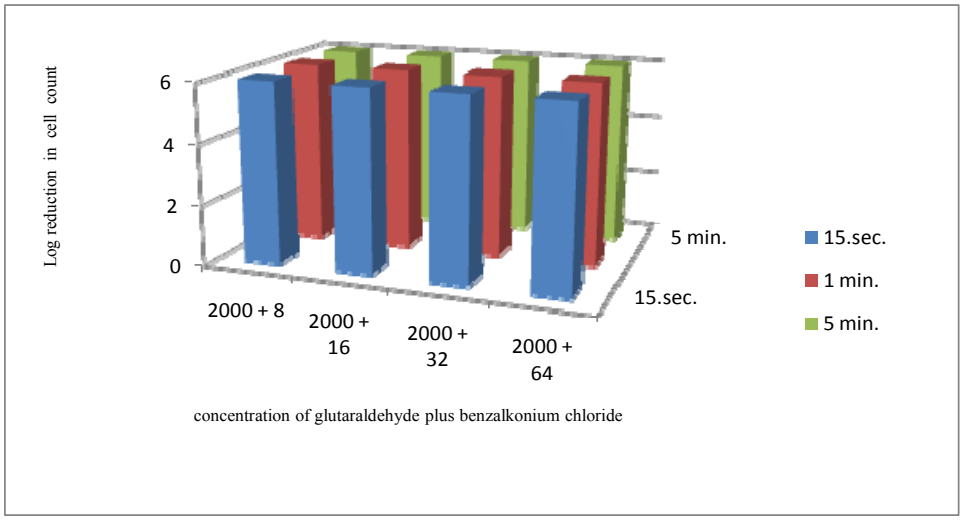


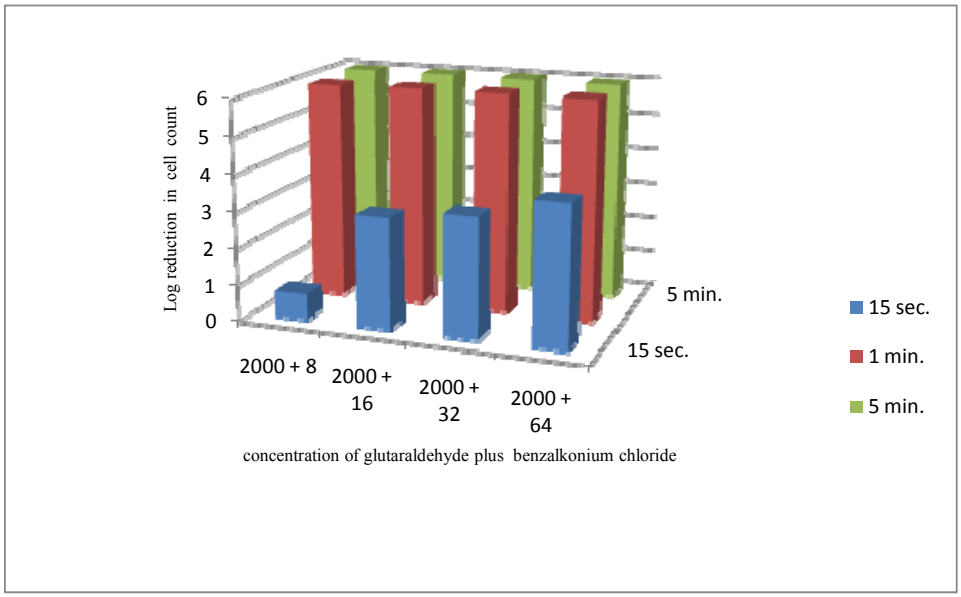
(b) *K. pneumonia*e

Fig. 3: Microbicidal effect of combination between glutaraldehyde and benzalkonium chloride as determined by the quantitative suspension test after exposure time 15 sec, 1 and 5 min.

Continued:



(c)) *P.aeruginosa*



(d) *S. typhi*

Fig. 3: Microbicidal effect of combination between glutaraldehyde and benzalkonium chloride as determined by the quantitative suspension test after exposure time 15 sec, 1 and 5 min.

Conclusion:

In conclusion, this study was to evaluate the efficacy of glutaraldehyde and benzalkonium chloride alone and in combination against antibiotic-resistant which had been collected of some clinical hospitals in Cairo, Egypt. The minimal inhibitory concentration (MIC) values of Glutaraldehyde and Benzalkonium Chloride, separately and in combinations was recorded.

The best $\log_{10}$ reductions of glutaraldehyde was concentration 3000 mg/L, while, benzalkonium chloride was concentration 512 mg/L for *all tested bacteria* after one minute contact. In the other hand, the preferred $\log_{10}$ reductions of combinations was 2000 mg/L of glutaraldehyde plus 8 mg/L of benzalkonium chloride after contact five minutes against *E. coli and S. typhi*, after one minute against *K. pneumonia*e and 15 second contact for *P. aeruginosa*.

From the results, we concluded that when glutaraldehyde and benzalkonium chloride were combined, it resulted in a synergistic action that enables the elimination of the bacterial isolates as easy task.

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