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The Impact of Diluted Detergents on *Escherichia coli* K12 (JM109)

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Abstract

The purpose of this study is to investigate the effect of diluted detergents namely, Clorox, Dettol and Aganol against *Escherichia coli* K12 (JM109), at different concentrations. Frequent usage of diluted detergents and disinfectants without knowing their effectiveness in killing microbes can act as a medium for infections in susceptible hosts. Moreover, it is known that the regular application of diluted detergents and disinfectants may actually cause antibiotic resistance. The efficacy of diluted detergents is determined by the minimum inhibition concentration (MIC) and the minimum bactericidal concentration (MBC) using tube dilution assay. The susceptibility test is conducted using the disc diffusion technique. Dettol and Clorox exhibited effective bacteriostatic agents where the MIC is 0.75%. The lowest concentration of Dettol and Clorox required to kill *E. coli* K12 (JM109) or MBC is at the concentration of 3.0% and 5.0%, respectively. Aganol showed less effective bacteriostatic and bactericidal agents, where it required higher MIC of 1.25% and MBC of 10%. The susceptibility test indicated that the Clorox is the most effective antibacterial agent with the minimum inhibition zone of 7 mm at a concentration of 1.75%. Higher concentrations of Dettol and Aganol (of 10% and 20%, respectively) are needed to exhibit the antibacterial activity with the minimum inhibition zone of 7 mm.

Keywords: Clorox; Dettol; Aganol; *E. coli* K12 (JM109); minimum inhibition concentration (MIC); minimum bactericidal concentration (MBC).

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1. Introduction

The wide employment of detergents and disinfectants in hospitals and medical centres to control growth and eliminate microbes is a fundamental part of infection control practices. Detergents and disinfectants are further applied for cleaning and sterilising clinical equipment. According to Russel (2003), the antimicrobial activities of disinfectants are influenced by the formulation of disinfectants, level of organic charge, temperature, dilution rate and methods used to determine antimicrobial activities. The susceptibility of different types of microorganisms to antiseptics and disinfectants vary from one bacteria to another [1]. In a review article by Russell [2], bacterial spores are the most resistant, followed by mycobacteria, Gram-negative bacteria and cocci.

Frequent usage of diluted detergents or disinfectants without knowing their effectiveness in killing the microbes may act as the cause for infections in susceptible hosts. It is also known that the frequent use of diluted detergents or disinfectants can cause antibiotic resistance. Some researchers indicated that the effect of disinfectants on microorganisms is concentration-dependent [3]. To investigate whether diluted disinfectants and detergents can cause antibiotic resistance, a link between antibiotics and the resistance of detergents and disinfectants in bacteria must be established. The resistance of the cell walls of the Gram-negative bacteria to dissociate by detergents, disinfectants and antiseptics is well acknowledged [4-7]. Nixdorff and his colleagues [5] suggested that the occurrence of tremendous amounts of lipopolysaccharides (LPS) and proteins, along with small amounts of phospholipid in the external surface of the exterior membrane, is the main factor for this resistance.

Clorox bleach is a solution containing 3-8% sodium hypochlorite (NaOCl), by weight [8]. NaOCl is normally used for the disinfection of surfaces [9]. It is extensively employed for hard-surface disinfectants, and can be utilised for cleaning leakages of blood containing human immunodeficiency viruses or HBV [7]. Dettol is an aromatic compound derived from phenol, which contains an important chlorine atom. Dettol is extensively applied in households and health-care surroundings for numerous reasons comprising of disinfection of the skin, objects, equipment, as well as, surrounding surfaces [10]. The antibacterial influence of Dettol is stronger against *Staphylococcus aureus*, *Salmonella typhi* and *E. coli* than against *Salmonella dysenteriae* and *Klebsiella sp* [11]. Aganol is an antibacterial used to clean floors.

The present study focused on the effect of diluted detergents (Clorox, Dettol and Aganol) against *E. coli* K12 (JM109), Gram-negative opportunistic bacterium. It is used in this study due to easy to handling and can be cultured in Biosafety Level 1 Microbiology Laboratory (involving low risk microbes). The minimum inhibition concentration (MIC), minimum bactericidal concentration (MBC) applying tube dilution assay and susceptibility test using the disc diffusion method were conducted in order to investigate the efficacy of the detergents at various concentrations against *E. coli* K12 (JM109).

2. Materials and Methods

2.1 Preparation of 0.5 McFarland turbidity standard and bacteria inoculum

McFarland 0.5 turbidity standard was prepared according to Microbeonline [12]. Three to four single colonies from the overnight bacterial culture were transferred to a tube of sterile normal saline to form the inoculum. The inoculum was resuspended to avoid lumps. The inoculum turbidity was compared to the McFarland 0.5 turbidity standard by holding them next to each other in front of a paper with black stripes printed on it. The inoculum should blur the black stripes to the same extent of the standard, or adjusted to match the standard.

2.2 Determination of MIC and MBC

The bacteriostatic activity was evaluated by determining the minimum inhibitory concentration (MIC). The lowest concentration (highest dilution) of the detergent preventing growth is considered to be the minimum inhibitory concentration (MIC). The minimal bactericidal concentration (MBC) of an antibacterial is defined as the maximum dilution of detergents that kill 99.99% of bacteria. MBC is determined by sub-culturing the MIC tube onto the growth medium without a detergent, and examining for bacterial growth. MBC must be performed and determined after the MIC experiment; whereby, 100 µL of the overnight MIC culture and cultures with detergent concentrations higher than MIC were inoculated into fresh nutrient broth (NB) without detergent. To determine the MIC, tube dilution assay was performed by constantly increasig the percent concentration of detergents into NB in a series of tubes. The detergents were diluted to the following concentrations: 0.25%, 0.5%, 0.75%, 1.0%, 1.25%, 1.5%, 1.75%, 2.0%, 2.5%, 3.0%, 5.0%, 10%, 20%, 40% and 80%. The undiluted detergent concentration was assumed as 100%. The diluted detergent concentrations were calculated according to the M1 V1 = M2 V2 formula, where M1 is the concentration of the undiluted detergent (100%), V1 is the volume of the 100% detergent used to achieve the M2 molarity, M2 is the diluted concentration that was tested (< 100%) in this study and V2 is the final volume (3000 µL). Hundred microliter of bacteria inoculum as prepared in 2.1 was added into each dilution tube except positive control tube. All experiments were performed three times with positive and negative controls. Positive controls were prepared as follows: nutrient broth medium without *E. coli* K12 (JM109) inoculum and 100% detergent with *E. coli* K12 (JM109) inoculum. No bacterial growth should be present in the positive control tubes. Negative control is defined as the presence of *E. coli* K12 (JM109) growth after overnight incubation at 37ºC in nutrient broth with 0% detergent. The MIC and MBC experiments were performed at 37ºC with overnight (12 hours) incubation at 120 revolutions per minutes (rpm). The MBC value was then verified by plating 100 µL of the overnight culture from the MBC tube onto the nutrient agar (NA) plate and incubated overnight at 37ºC. No bacterial growth should be detected on the overnight NA plate.

2.3 Sensitivity test of disc diffusion activity

Mueller Hinton agar (MHA) was used for the susceptibility test by the Kirby-Bauer disk diffusion technique [13]. The discs (6 mm in diameter) were prepared by perforating the filter paper, Whatman No.3, using a paper puncher and then sterilised at 121°C for 15 minutes. A sterile cotton swab was dipped into the overnight broth culture of *E. coli* K12 (JM109), raised above the fluid level and rotated within the tube to remove excess liquid. Next, the swab was streaked onto the entire surface of the MHA plates. The plates were left to dry for 10 minutes at 37ºC before applying the discs. The discs were immersed into the diluted detergents of the following concentrations: 0.25%, 0.5%, 0.75%, 1.0%, 1.25%, 1.5%, 1.75%, 2.0%, 2.5%, 3.0%, 5.0%, 10%, 20%, 40% and

80%. Next, the discs were placed on the MHA that was inoculated with *E. coli* K12 (JM109) and incubated overnight (12 hours) at 37°C. A positive control consists of a disc immersed in 95% ethanol, and a negative control consists of a disc immersed in sterile distilled water. The inhibition zone was determined after the overnight incubation at 37ºC. The experiment was performed in triplicate. Mean and standard deviation (SD) of the inhibition zone values were calculated using the Microsoft Excel 2013 Software (Microsoft Corp., Redmond, WA).

3. Results

The concentration of 0.1% and 0.2% of Clorox, Dettol and Aganol displayed growth after overnight incubation at 37°C; thus, all detergents were tested at the initial diluted concentration of 0.25%. As shown in Table 1, the MIC of the Clorox against *E. coli* K12 (JM109) is at the concentration of 0.75%. The amount of microbe growth was indicated by the turbidity, where the least turbid or clear tubes (tubes with concentrations of 0.75% and above) correlate with the absence of microbes. The negative control is the tube without detergents which exhibited the most turbidity since the microbes were able to grow. Increases of the antimicrobial concentration decreases the turbidity until the MIC is reached; thus, microbes can no longer survive. Detergents that exhibit low MICs are more effective than those with high MICs since only low concentrations are needed to eliminate microbes.

Table 1: MIC determination of the diluted Clorox against *E. coli* K12 (JM109). T is referring to turbid and C is referring to clear broth

Concentration (%) of the NaOCl	Turbidity
0.25	T
0.5	T
0.75	C
1.0	C
1.25	C
1.5	C
1.75	C
2.0	C
3.0	C
5.0	C
10	C
20	C
40	C
80	C
-ve control (no detergent)	T
+ve control (only NB)	C
+ve control (undiluted Clorox)	C

The MIC determination of Dettol against *E. coli* K12 (JM109) was indirect. At the concentrations of 0.75% to 40%, the growth medium became turbid once Dettol was added to the medium. However, at the concentrations of 0.25%, 0.5% and 80%, Dettol did not cause the medium to become turbid. After overnight incubation, all tubes became turbid except the one with Dettol concentration of 80%. To determine which tube is the MIC tube, 100 μL of the overnight culture from tubes with Dettol concentrations of 0.75% to 40% were added to the

growth medium with no Dettol and incubated at 37°C for 12 hours. The MIC was identified at the Dettol concentration of 0.75% since this is the first tube to appear clear. The MIC of Aganol was much higher compared to Dettol and Clorox. The MIC of Aganol against *E. coli* K12 (JM109) was at the concentration of 1.75%. The overnight cultures from the MIC experiments were subjected to MBC experiments. The overnight culture tube with 0.75% to 3.0% Clorox concentrations were turbid, thus indicating bacterial growth. The MBC was detected in a clear culture tube with 5% Clorox concentration. To confirm that the MBC of the Clorox is 5%, 100 µL of the culture suspension from tubes with concentrations of 5.0% and 10% were plated on nutrient agar and incubated overnight at 37°C. No bacterial colonies were observed from the suspension of the tubes with concentrations of 5% and 10%. The MBC of Dettol could not be identified from the MBC tube dilution assay experiment since all broths were clear after overnight incubation at 37ºC. The MBC of Dettol was determined by plating 100 µL of clear tubes from the MBC experiment on to the NA plate. The MBC of Dettol was determined to be 3.0% concentration since no bacterial colonies were observed at this concentration. Aganol exhibited the highest MBC with the concentration of 10%. The Aganol MBC concentration value was validated with the NA plating experiment. The effectiveness of detergents with different concentrations against *E. coli* K12 (JM109) was determined by the disc diffusion plate method. The results were tabulated in Table 2. The Clorox showed the highest inhibition zone against *E. coli* K12 (JM109) of 41 mm ± 1.0. The inhibition zone measurements decrease with the increase of the Clorox dilution to the least inhibition zone of 7 mm at the concentration of 1.75%. No inhibition zones were detected at Clorox concentrations of 0.25%, 0.5%, 0.75%, 1.0%, 1.25% and 1.5%. The minimum inhibition zone of 7 mm was detected with 10% of Dettol and 20% of Aganol. Undiluted Dettol and Aganol with a concentration of 100% produced maximum inhibition zones of 17 mm ± 0 and 13 mm ± 0, respectively.

Table 2: The inhibition zone detected in susceptibility test of the diluted detergents (Clorox, Dettol, Aganol) against *E. coli* K12 (JM109

Concentration (%)	Inhibition zone (mm)		
	Clorox	Dettol	Aganol
0.25	Not detected	Not detected	No detected
0.5	Not detected	Not detected	Not detected
0.75	Not detected	Not detected	Not detected
1.0	Not detected	Not detected	Not detected
1.25	Not detected	Not detected	Not detected
1.5	Not detected	Not detected	Not detected
1.75	7.0 ± 0	Not detected	Not detected
2.0	7.5 ± 0	Not detected	Not detected
2.5	8.0 ± 0.5	Not detected	Not detected
3.0	8.5 ± 0.5	Not detected	Not detected
5.0	10 ± 0	Not detected	Not detected
10	11 ± 0.5	7.0 ± 0	Not detected
20	19 ± 1.0	9.0 ± 0	7.0 ± 0
40	26.6 ± 0.57	11 ± 1.0	8.0 ± 0
80	39 ± 0	15.6 ± 0.57	11.6 ± 0.57
100 (+ve control)	41 ± 1.0	17 ± 0	13 ± 0
Distilled water (-ve control)	Not detected	Not detected	Not detected

4. Discussion

The contamination of surfaces and tools are the main sources for the spread of pathogenic microbes. Therefore, the detection of appropriate concentrations of antimicrobial agents for the disinfection of contamination is of great practical value. The main objective of the present study was to evaluate suitable concentrations of detergen ts to inhibit and eliminate the growth of bacteria to avoid con tamination and the spreading of diseases. At the right concentration, biocides such as disinfectants and antiseptics are used to kill bacteria and microbes. However, the bacteria can survive and become resistant to treatment if lower levels are used. It is known that hospital-acquired infections are contributed by the bacteria that are resistant to disinfectants and antibiotics. These resistant bacteria build up is due to repeated exposure to the biocides [14]. The resistant mutants exhibits increased number of efflux pumps in the bacteria. Efflux pumps are transport proteins found in Gram-positive and -negative bacteria for transporting out toxic substrates including antibiotics of multiple classes from within cells into the external environment. Thus patients who are infected with pathogenic bacteria with more pumps could be more resistant to treatment [14]. All tested detergents displayed antibacterial activity against *E. coli* K12 (JM109) with specific MIC and MBC values that reflect the unique properties of the detergents' chemical structures. In this study, Dettol's MIC and MBC were 0.75% and 3.0%, respectively; while Clorox's MIC and MBC were 0.75% and 5.0%, respectively. Aganol was less effective against *E. coli* K12 (JM109) when compared to Dettol and Clorox where the MIC was 1.25% and the MBC was 10%. The minimum inhibition zone of Clorox, Dettol and Aganol was 7 mm at concentration dilutions of 1.75%, 10% and 20%, respectively. The inhibition zones of undiluted Clorox, Dettol and Aganol with 100% concentrations were 41 mm ± 1, 17 mm ± 0 and 13 mm ± 0, respectively. Chlorine- and iodine-based compounds are the most important microbicidal halogens that are used in the hospitals and have been conventionally used for the purposes of antiseptics and disinfectants. The best and significant types of chlorine releasing agents (CRAs) are sodium hypochlorite. These are highly active oxidizing agents and thereby destroy the cellular activity of proteins [15]. Sodium hypochlorite (NaOCl) ionizes in water to produce sodium cation (Na+) and a hypochlorite anion(ClO−); which establishes an equilibrium with hypochlorous acid, HOCl [16]. The active moiety chlorine exists predominantly as HClO between pH 4 and 7, whereas above pH 9, OCl_2 predominates. Disruption of oxidative phosphorylation and membrane-associated activity by HOCL have also been reported [17, 18]. Hypochlorous acid inhibits bacterial growth by inhibiting the DNA synthesis. This was shown in a study where at 50 mM (2.6 ppm), *E. coli* was completely inhibited within 5 minutes. The inhibition was due to the halting of DNA synthesis by 96% and protein synthesis by 10 to 30%. On the other hand, at concentrations below 5 mM (260 ppm) bacterial membrane disruption or extensive protein degradation were not induced thus indicated that DNA synthesis was the sensitive target [19]. When the chlorine solution was used during mechanical ware-washing, the reduction of *E. coli* K-12 from the plates is likely to be higher [20]. According to a report [21], sodium chlorite possess the greatest influence against *E coli* in 10% concentration. In another study, it was reported that 0.5% of bleach killed almost 80% of bacteria, whereas 1% and 5% of bleach kills 100% of micro-organism [22]. Dettol or 4-Chloro-3,5-dimethylphenol (chloroxylenol) are those kind of chemicals that are immediately identified by its characteristic odor. It is an aromatic compound derived from phenol, which contains an important chlorine atom. *P. aeruginosa* and many molds are highly resistant to chloroxylenol although it is bacterical [23, 24]. Chloroxylenol has been used widespread over many years but little studies has been done on the mechanism of

action. However, it would be expected to target microbial membranes due to its phenolic nature. Dettol is extensively used in households and health-care surroundings for numerous reasons comprising of disinfection of skin, objects, and equipment, as well as surrounding surfaces. With previous scrubbing before application, the number of micro-organisms inhabiting the skin and surfaces are seriously reduced [8]. The antibacterial influence of Dettol was stronger against *Staphylococcus aureus*, *Salmonella typhi* and *E. coli* than against *Shigella. dysenteriae* and *Klebsiella sp* [25]. According to Thomas and his colleagues [26], the MIC and MBC of Dettol as disinfectant for *E coli* is 2%, while for the wound pathogenic bacteria the MIC was 1.56% and MBC was 3.13%. Rasha and his colleagues showed that 3 types of different chloroxylenol (termed S1, S2, Sp) from different companies exhibited different MIC value against *P. aeruginosa, E. coli*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *B. subtilis* [27]. The S1 chloroxylenol exhibited MIC value of ~ 500 µg/ml for *P. aeruginosa* and *E. coli* whereas for MRSA and *B. subtilis* is ~125 µg/ml. The S2 chloroxylenol exhibited MIC value of 2000 µg/ml, 4000 µg/ml, 250 µg/ml and 125 µg/ml for *P. aeruginosa, E. coli, MRSA* and *B. subtilis*, respectively. The Sp chloroxylenol showed no significant effect against *P. aeruginosa* and *E. coli* but inhibited MRSA and B. *subtilis* with the MIC value of 2048 µg/ml and 1024 µg/m, respectively. Earlier study by Dawaf, demonstrated that MIC values of chloroxylenol for *P. aeruginosa, E. coli and S. aureus* were 1024, 1024 and 128µg/ml respectively [28]. On the other hand, Davies and his colleagues showed that chloroxylenol was less active against *P. aeruginosa* but has good activity against Gram-positive than Gram-negative bacteria [29]. Aganol or alkylbenzyldimethylammonium chloride is an antibacterial floor cleaner. It eliminates 99.9% of undesirable germs while refreshing the room with a floral or fruity fragrance [30]. To date no publications were reported on inhibition of bacteria using Aganol. Alkylbenzyldimethylammonium chloride is a quaternary ammonium compound (QAC) that is widely used as disinfectants, fabric softening agents, foam depressants, and antistatic agents that lead to massive discharge into the environment with its associated concerns [31]. In addition, several studies have been reported that repeatedly exposure to QACs can increased the risk of developing microbial resistance against antibiotics in many pathogenic microorganisms [7]. Bacterial resistance to antiseptics and disinfectants can be either a natural property of an organism (intrinsic) or acquired by mutation or acquisition of plasmids (self-replicating, extrachromosomal DNA) or transposons (chromosomal or plasmid integrating, transmissible DNA cassettes) [7]. The term “resistance” based on MIC analysis should be interpreted carefully especially for MIC biocides. As for antibiotics, an increase in the MIC may indicated that the antimicrobial action is not acting on the target organisms. However, an increased of MIC biocides may not necessary means failure of the therapeutic. It is necessary to take into considerations the following factors; formulation effects, direct product application, concentration used in products, and bactericidal activity before making any conclusion on the clinical implications.

5. Limitation of the study

Although this research was carefully designed and has reach it aims; there were some unavoidable limitations. In this study only *E. coli* K12 (JM109) was used because the laboratory is qualified only for Biosafety level 1. Thus, to make generalization for pathogenic Gram-positive and -negative bacteria, this study should involve a larger sample of bacteria normally found in the household and hospital environments. Second, the short period of time for conducting this study limit our findings on the development of the bacteria resistant to some antibiotics due to the frequent exposure to disinfectant.

6. Conclusions and Recommendations

All detergents (namely, Cloroxl, Dettol and Aganol) exhibited antimicrobial activities against *E. coli* K12 (JM109) at various diluted concentrations. The Clorox and Dettol were the most effective antibacterial agents when compared to Aganol, as indicated by the MIC, MBC and susceptibility results. To further evaluate the effect of the frequent exposure of the diluted disinfectants towards the development of the bacteria resistant to some antibiotics, this study need to be extended. More antiseptics and disinfectants should be tested in this study in order to observe the resistant trends that might be developed under different types of diluted biocides. The MIC and MBC of the diluted biocides for larger sample of pathogenic bacteria found in households and hospitals need to be determined. These pathogenic bacteria need to be repeatedly exposed to the diluted biocides and followed with the MIC determination. Then, the exposed bacteria with an increased in the MIC value will be tested for antibiotics resistance using disc diffusion and MIC techniques. The MIC antibiotics of the exposed bacteria must be compared with the MIC of the non-exposed bacteria Lastly, to understand how the antibiotics resistance develops in these bacteria, a study on the ion channels and the resistant mechanism have to be conducted.

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