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Detection of Microbial Contamination in Some Lebanese Schools

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A B S T R A C T

A comparative study of indoor contamination in three private and three official schools in Aley, Lebanon was conducted during the spring season of the year 2014. Sampling was performed in each school, from the air, desk-surfaces of classes and water taps of bathrooms. The samples were examined for microbial contamination and the bacterial colony-forming units (CFU) for each site were enumerated. Official schools showed to be highly contaminated (air mean 87 CFU and surface mean 98 CFU) with respect to the private schools (air mean 61 CFU and surface mean 60 CFU). Among the three educational levels, the elementary level was the most contaminated. Fungal contamination was also detected on classes' desks and three genera were morphologically identified; *Aspergillus*, *Penicillium* and *Cladosporium*. Eighty morphologically different bacterial isolates were obtained, among which Gram-negative bacteria (52.5 %) were encountered slightly more than Gram-positive bacteria (47.5 %). These isolates were tested for their susceptibility to six commonly used biocides in indoor decontamination; Ajax®, Clorox®, Ethanol 70 %, Dettol®, Easy® and CAMEO®**,** by well diffusion test. The highest frequency of resistance amongst the eighty bacterial isolates was detected against Easy® (93.75 %), while the lowest frequency was detected against Clorox® (46.25 %). Moreover, the susceptibility of the biocides was in the order: Clorox® > Dettol® > Ajax® > CAMOE® > Ethanol > Easy®. Seven bacterial isolates, which showed reduced susceptibility to three or more biocides were selected and identified. The minimum inhibitory concentration and the minimum bactericidal concentration of the six tested biocides were evaluated against selected isolates, they ranged from 200 µl/ml to 900 µl/ml. All MIC indexes calculated were less than 4 which displayed the bactericidal activity of the six biocides against the seven tested bacterial strains. Isolate 51 (*E. coli*) was the least susceptible strain to biocides among the seven selected isolates, the results of the time kill assay revealed that the time needed to kill this isolate by Dettol® was 8 min., while other biocides took longer time to kill it. In addition, the loss of resistance of *E. coli* against the six biocides was recorded after plasmid curing, which proved the plasmid-mediated resistance.

Introduction

In the indoor environment, we encounter microorganisms on virtually every surface we touch, and this frequent exposure to indoor microbes carries with it the potential for disease transmission, as well as interactions with our own commensal microbiome (Meadow et al., 2014).

Microbiological studies have shown that viable microorganisms, including bacteria and fungi, are readily obtained from various indoor environments **(**Kämpfer et al., 1999; Flores et al., 2011; Awe et al., 2013**)**. Culture-based studies of human indoor environments have shown that significant levels of bacteria are present in seemingly innocuous areas such as office buildings, residential homes, shopping centers, children's schools and daycare centers (Lee et al., 2007).

School facilities are densely populated, so the maintaining of good quality indoor environments is a difficult problem (Karwowska, 2003). Disinfectants and antiseptics are freely available without prescription and they are widely used as part of infection control practices and in the prevention of nosocomial infections (Smith and Hunter, 2008). The increased usage of these biocides has raised the emergence of microbial resistance.

This problem displays the importance of developing new strategies to limit the different mechanisms of resistance. Previously, many investigations have been carried out to study the bacterial resistance to antibiotics, but the limited data available on biocide resistance of bacteria in school environments highlighted the need for this investigation. Therefore, the aim of the present study was to evaluate and compare the microbial contamination in six Lebanese school environments; three official and three private, as well as the bacterial isolates' susceptibility to commonly used biocides in indoor decontamination was also investigated. To the best of *our knowledge*, this was the *first study* in Lebanon addressing the issue of biocide resistant bacteria in the school environment.

Materials and Methods

Isolation of Microbial Contaminants from School Environments

The isolation of bacterial contaminants was carried out during spring 2014 from three official schools (Kayfoun Official School, Bennay Official Schools and Mejdlayya Official School) and three private schools (High National College, Najah School and New Steps College), all schools are located in Mount Lebanon-Aley. In each school, collection of air and surface samples was performed from 12 classes; from Kindergarten 1 till Grade 9 and two bathrooms; a girl's and a boy's bathroom. Surfaces (25 cm^2) were swabbed using a sterile cotton swab moistened with sterile distilled water. Swabs were used to inoculate Nutrient Agar (NA), MacConkey Agar (MCA) and Sabouraud Dextrose Agar (SDA) within 10 minutes of collection.

Air collection was simultaneously performed by exposing nutrient agar plates to air of a closed and unoccupied room for 30 minutes. The sampling procedure was performed in triplicates in the middle of the school day. NA and MCA were examined after 24 hrs of incubation at 30°C, whereas SDA plates were examined after 7 days of incubation at 30°C. The average number of bacterial colony-forming units (CFU) for each site was enumerated. Fungal and bacterial isolates were purified and maintained on SDA and NA, respectively.

Phenotypic Characterization of Microbial Isolates

Eighty bacterial isolates were differentiated based on the morphological characteristics of colonies (diameter, color, surface, pigmentation, edges and elevation), as well as the Gram stain of bacterial cells. Seven bacterial isolates (11, 30, 44, 51, 52, 57 and 58) that show reduced susceptibility to 3 or more biocides were identified using API-test kits (BioMérieux, France).

Fungal isolates were stained with Lactophenol blue and examined under the microscope.

They were identified morphologically to the genus level according to the structure of hyphae, conidiophores and conidia.

Biocides Susceptibility Testing of Bacterial Isolates

Biocidal products are defined as active substances, which can destroy, render harmless and prevent the multiplication of harmful microorganism (Maillard, 2005). Six commonly used biocides for surface cleaning in schools were purchased in commercial preparations from local supermarkets. The eighty selected bacterial isolates were tested for their susceptibility to six commonly used biocides in indoor decontamination; Ajax®, Clorox®, Ethanol 70 %, Dettol®, Easy® and CAMEO®, by well diffusion test and the average diameters of inhibition zones formed were measured. Standard bacterial inocula equivalent to 0.5 McFarland were used for plate's inoculation. Inhibition zones less than 10 mm in diameter were recorded for resistant isolates while zones more than 16 mm were recorded for susceptible strains (Adenike et al., 2011; Okesolab and Olola 2011). The minimal inhibitory concentration and the minimal bactericidal concentration of biocides against bacterial isolates, that showed reduced susceptibility to 3 or more biocides, were evaluated. Series of doubling dilutions, using Müeller-Hinton Broth, of each tested biocide, were prepared in 96-well microtiter plates and inoculated with standard inocula for MIC determination. The dilutions of the MIC experiment showing no visible growth were inoculated into Müeller–Hinton agar plates, then MBCs were determined as the lowest concentrations resulting in no growth. Cultures were incubated at 30° C for 24 hrs. The MIC index (MBC/MIC) was calculated for the six biocides to determine whether each biocide had bactericidal (MBC/MIC < 4) or bacteriostatic (MBC/MIC > 4) effect on the growth of tested bacterial strains **(**Chattopadhyay et al., 2007**)**. Time–kill curves were constructed for the most resistant bacterial strain (isolate 51) using MBC values of the tested biocides and the optical density was measured at 600 nm at different time intervals.

Biocides Susceptibility Testing of the Plasmid Cured Isolate

Most of the genetic determinants that confer resistance to antimicrobials are located on plasmids and some are located on the chromosome **(**Maiden, 1998). Bacterial plasmids are known to harbor genes for resistances to antibiotics and metals **(**Ghosh, et al., 2000). Bacterial plasmids can be eliminated from bacterial species by several methods (Yamamoto et al., 1988; Russell, 1997; Heir et al., 1998, 1999). Bacterial plasmids, in the current study, were cured by exposing the cells to ethidium bromide. The least susceptible strain to biocides (isolate 51) was plasmid cured and retested again for biocides susceptibility using well diffusion test.

Statistical Analysis

Microsoft Excel® was used to enter and capture data. Various graphs and tables were extracted from this data. Data was then exported to Statistical Package for the Social Sciences (SPSS) for further analysis. Results of CFU in the schools were analyzed using one-way analysis of variance (ANOVA). P value < 0.05 was considered as significant. IBM SPSS® version 20 was employed for statistical analysis.

Results and Discussion

Microbial Contamination of the School Environments

A total of 14 surface samples (12 class desk surfaces and 2 bathroom water taps) and 14 air samples (12 air class samples and 2 bathroom air samples) extending amongst the classes and bathrooms of each of the six schools, were tested for the evaluation of microbial contamination.

Official schools showed higher contamination levels than private ones. The relation between the school type and bacterial contamination was significant ($p < 0.05$) for both the air and the surface samples (Table 1). In addition, the relation between the educational levels and bacterial CFU was highly significant in both the air and the surface samples ($p < 0.05$) (Table 2).

Identification of Microbial Isolates

Eighty morphologically different bacterial strains were isolated, among which Gram-negative bacteria (52.5 %) were encountered slightly more than Gram-positive bacteria (47.5 %). The percentages of Gram-positive cocci, bacilli and coccobacilli were 17.5 %, 16.25 %, and 13.75 %, respectively. Gram-negative bacterial isolates were bacilli only. Twenty-one fungi were isolated from desk surfaces and were morphologically identified to the genus level. Fourteen *Aspergillus* species, four *Penicillium* species and three *Cladosporium* species were detected.

Biocide Susceptibility Testing

The six biocides (Ajax®; Clorox®; Ethanol; Dettol®; Easy®; CAMEO®) under investigation were tested against the eighty bacterial isolates by the well diffusion susceptibility test. The highest percentage of resistance amongst the eighty bacterial isolates was seen against Easy® (93.75 %). Furthermore, 87.5 %, 66.25 %, 56.25 % and 53.75 % of the isolates showed resistance to ethanol, CAMOE®, Ajax® and Dettol®, respectively. The lowest frequency of resistance among all isolates was detected against Clorox® (46.25 %). Thus, the susceptibility of the isolates against tested biocides was in the order Clorox® > Dettol® > Ajax® > CAMOE® > Ethanol > Easy® (Fig. 1).

Selection and Identification of Bacterial Isolates with Reduced Susceptibility to Biocides

Seven bacterial isolates (11, 30, 44, 51, 52, 57 and 58), that showed reduced susceptibility to three or more biocides, were selected for testing the susceptibility against biocides under investigation. The most active biocide against isolate 11, 44, and 52 was Clorox® with average inhibition zone diameters of 16, 15, and 15 mm, respectively. Dettol® was the most effective agent on the growth of isolate 30, 51 and 57 with average inhibition zone diameters equaled to 15, 14 and 16 mm, respectively. Finally, isolate 58 was most affected by Ajax® with 15 mm average inhibition zone diameter.

The identification of the seven isolates was conducted using the API test kit. Isolates number 11, 44 and 58 were *Staphylococcus epidermidis* and isolates number 30, 51, 52 and 57 were *Escherichia coli.*

MIC and MBC of the Tested Biocides Against Selected Isolates

The MIC and MBC values determined for the tested biocides against the seven selected isolates (11, 30, 44, 51, 52, 57 and 58) ranged from 200 µl/ml to 900 µl/ml. All MIC indexes calculated were less than 4 which displayed the bactericidal activity of the six biocides against the seven tested bacterial strains. Isolate 51 was killed by the highest values of MBCs, which were recorded for the two biocides; ethanol and Easy® (900 µl/ml).

Time-Kill Curves of the Isolate 51

Based on the previous data, isolate 51 (*E. coli*) showed the least susceptibility to different biocides as well as elevated MBC values. Isolate 51 was selected for testing the time course of killing activity of the most effective biocides having the lowest MIC and MBC values. The time taken to kill *E. coli* by Ajax® (16 min) (Fig. 2) was longer than the time taken to kill it by Clorox® (14 min) (Fig. 3) and Dettol® (8 min) (Fig. 4).

Biocides Susceptibility Re-testing of the Plasmid Cured Isolate 51

After plasmid curing, isolate 51 showed increased susceptibility to all the tested biocides that was indicated by an increase in the inhibition zone diameter. For Ajax, the inhibition zone diameter increased from 10 to 17 mm (1.7 fold increase), from 13 to 29 mm for Clorox®, from 7 to 22 mm for Ethanol, from 14 to 25 mm for Dettol®, from 7 to 22.5 mm for Easy®, and from 8 to 16 mm for CAMEO® (Fig. 5). Thus, the loss of resistance of *E. coli* against the six biocides was recorded after plasmid curing, which proved the plasmid-mediated resistance.

The highest contamination level was ascribed to the number of students, the location and the environment surrounding the school's buildings and the cleaning protocols in the schools. The bacterial CFU recorded displayed the high surface contaminants that were encountered in the official schools (98 CFU) in comparison with that in private schools (60 CFU). This was linked to the high number of Syrian refugees' students who enrolled to the official schools during the academic year 2013-2014. These students lacked suitable hygiene attitudes due to their low economic level. Among the three educational levels, kindergarten level had the least mean of surface CFU (48 CFU) with respect to the elementary and intermediate levels; which were 108 CFU and 73 CFU, respectively. Hence, kindergarten students in all the studied schools had toilet access assistantship and better hygiene applications that were monitored by their teachers.

It was observed that the prevalence of Gram-negative bacteria (52.5 %) were encountered slightly more than Gram-positive bacteria (47.5 %) in the eighty environmental samples originating from the different sites in the six schools. This finding was not in accordance with the study of Jomha et al (2014) that showed Gram-positive bacteria (82.7 % of isolates) were encountered more often than Gram-negative bacteria (17.3 %) in a Lebanese tertiary care hospital. In the current study, all Gram-negative bacteria were bacilli. Among the Gram-positive isolates, Gram-positive cocci were the most frequent (17.5 %); while the

prevalence of bacilli and coccobacilli, was 16.25 %, and 13.75 %, respectively. Moreover, the most common fungal isolate was *Aspergillus* (67 %) followed by *Penicillium* (19 %), then *Caldosporium* (14 %). This result coincided with the work of Leite et al (2012) that showed that the genus *Aspergillus*. (89.6 % of the samples) was highlighted as one of the principal fungi present in indoor environments followed by genus *Penicillium* (10.4 % of the samples). Also, a survey of airborne fungi in buildings and outdoor environments in the US (2002) found that *Aspergillus* spp. was the most common fungi (Shelton et al., 2002).

Seven bacterial strains that showed reduced susceptibility to three or more tested biocides, were selected and identified. Four out of the seven bacterial isolates (isolates number 30, 51, 52, and 57) were *Escherichia coli*. *E. coli* is commonly found in the environment and is usually an indicator of fecal contamination **(**CDC, 2006). Schools, which had previously reported outbreaks of gastro-intestinal infections, had higher levels of fecal contamination on children's hands than schools, which had not reported outbreaks. Hand contamination, contamination of sites such as taps and sinks, and classroom objects were found to be significant predictors of diarrhea risk. Ways to reduce these contaminations need to be explored including greater emphasis on hygiene education and monitoring of hand washing. Kaltenthaler et al (1995) concluded that during outbreaks of diarrhea, hands and classroom objects play a role in the transmission of diarrhea in day care centers.

Three out of the seven identified bacterial isolates (isolate number 11, 44, and 58) in the current investigation were found to be *Staphylococcus epidermidis.* Meadow et al (2014) were also detected *Staphylococcus epidermidis* on chairs of classroom, which are always in contact with human skin. *S. epidermidis* often colonizes the skin and mucous membranes of the human body, representing an important part of its normal microflora. It has the ability to adhere to biomaterials surface and develop as biofilm, which constitutes an important virulence factor and the most important pathogenic mechanism of staphylococcal infection **(**Löwdin et al., 1998).

Chlorine, the most common disinfectant, is desirable because it has a wide spectrum of use, fast acting and inexpensive (Lawrence and Bennet, 2001) and it is moderately oxidative and reacts with various components of bacterial cells (Kim et al., 2008). In the present investigation, the lowest frequency of resistance among all isolates was detected with Clorox® (46.25 %). It showed the most inhibitory effect against isolates 11 and 44 (*Staphylococcus epidermidis*), and 52 (*Escherichia coli*), with an average inhibition zone diameters of 21, 15, and 15 mm, respectively. This was in accordance with Kim et al (2008) who showed that chlorine was the most effective among three tested antimicrobial agents against planktonic cells.

Chimezie et al (2013) mentioned that Dettol® was the most effective in inhibiting the four organisms tested; *Staphylococcus* spp., *Bacillus* spp., *Escherichia* spp. and *Pseudomonas* spp.. They obtained inhibition zone diameter of 15 mm for both *Staphylococcus* spp. and *Escherichia* spp. against Dettol®. In our study, 46.25 % of the isolates showed susceptibility to Dettol®, with average inhibition zone diameters of 15, 14 and 12 mm for *Staphylococcus epidermidis*; isolates 11, 44 and 58, respectively and average inhibition zone diameters of 15, 14, 13 and 16 for *Escherichia coli*; isolates 30, 51, 52 and 57, respectively.

Table.1 Mean of Bacterial CFU of Air and Surfaces in Official and Private Schools

School Type / Sampling Site	Official Schools	Private Schools	F value
Air Mean	**87**	**61**	**1014****
Surface Mean	**98**	**60**	**2166****

**Highly significant at $p < 0.05$

Figure.1 Frequency of the Less Susceptible Isolates against the Six Tested Biocides

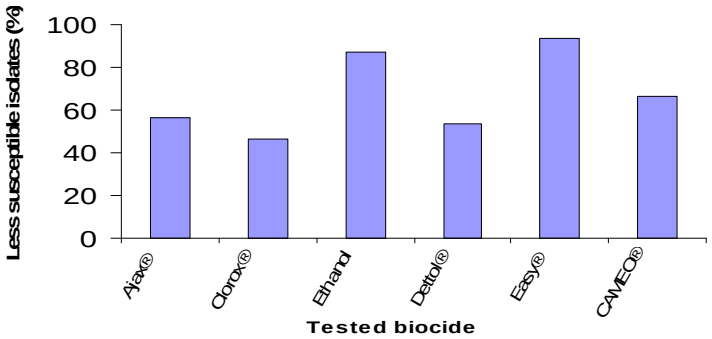

Figure.2 Survival Curves of *E. coli* (isolate 51) Treated with Ajax® Compared with Untreated

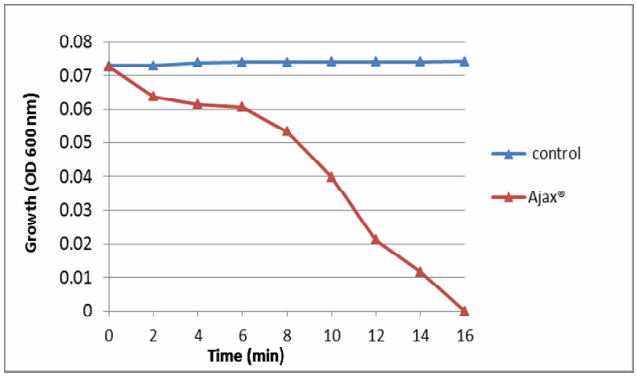

Figure.3 Survival Curves of *E. coli* (isolate 51) Treated with Clorox® Compared with Untreated

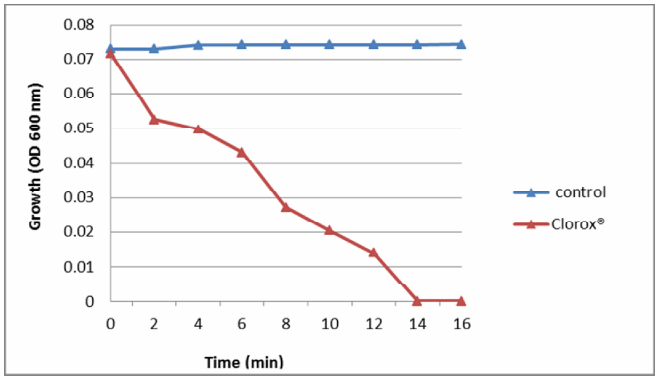

Figure.4 Survival Curves of *E. coli* (isolate 51) Treated with Dettol® Compared with Untreated

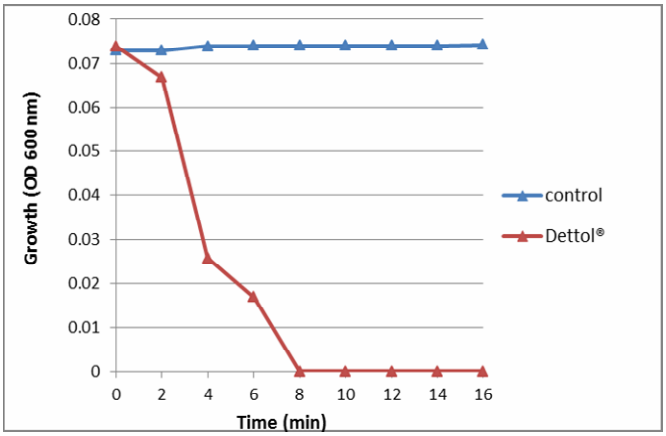

Figure.5 Antibacterial Activity of the Biocides (a. Ajax®, b. Clorox®, c. Ethanol 70%, d. Dettol®, e. Easy® and f. Liquid Soap: CAMEO®) against *E. coli* before and after Plasmid Curing

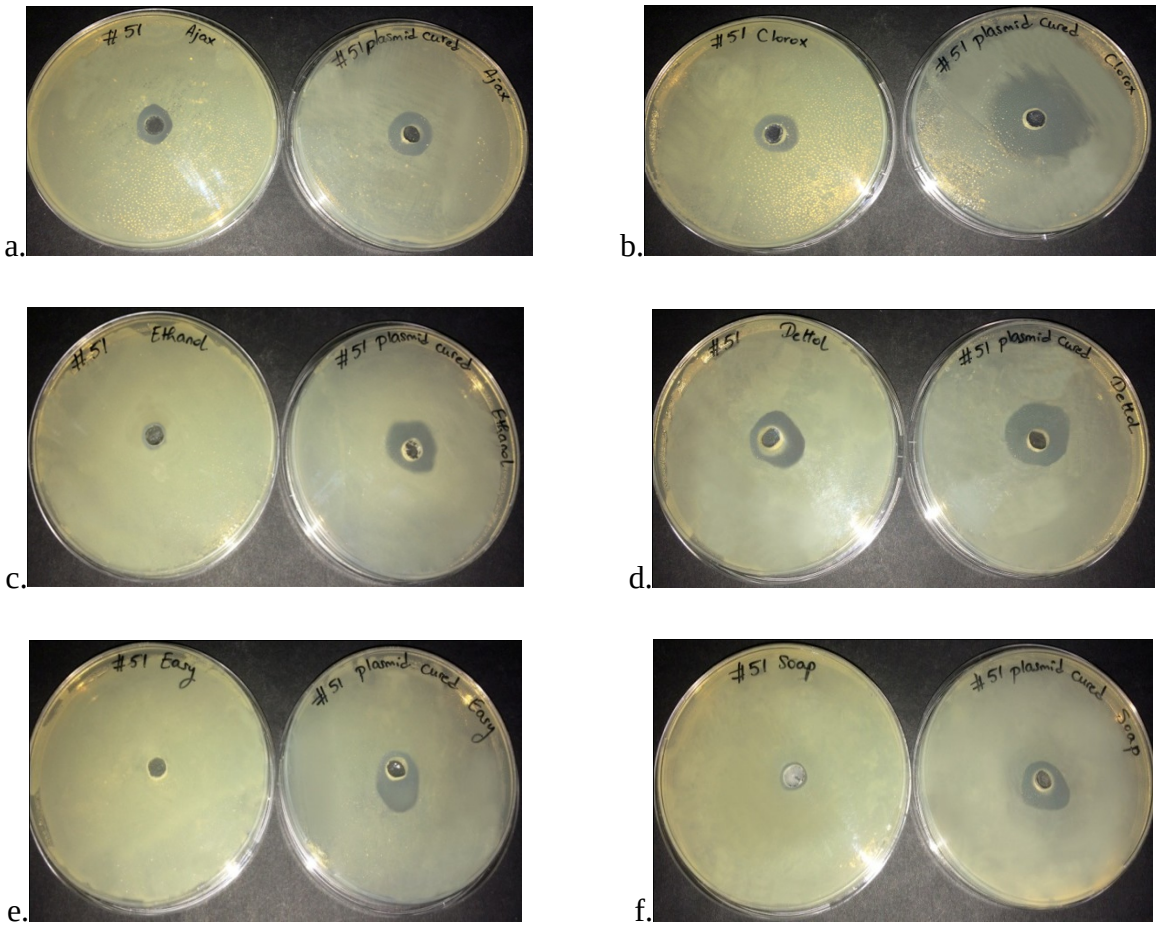

Table.2 Mean of Bacterial CFU of Air and Surfaces in the Three Educational Levels of all Schools

Educational Level / Sampling Site	Kindergarten	Elementary	Intermediate	F value
Air Mean	**62**	**95**	**70**	**889****
Surface mean	**48**	**108**	**73**	**2725****

**Highly significant at $p < 0.05$

Isolate 51 (*E. coli*) showed the least susceptibility among the seven identified isolates to tested biocides, as well as elevated MBC values. Time-kill curves constructed for treated 51 isolate with the disinfectants showed that the highest exposure time sufficient to kill it (16 minutes) was recorded by the application of Ajax. According to Chimezie et al (2013), the higher the value of killing rate, the faster the efficiency of the killing process. They found that the killing rates of *Staphylococcus* spp. and *Escherichia* spp. by Dettol® were higher than for other disinfectants. This is in accordance with the current obtained results, which proved that the time taken to kill *E. coli* (isolate 51) by Dettol® (8 min) was shorter than the time taken to kill it by Clorox® (14 min) and Ajax® (16 min).

Plasmid-mediated resistance to biocides is a well recognized phenomenon and bacterial plasmids are known to harbor genes for resistances to antibiotics (Mitchell et al., 1998; Ghosh et al., 2000). Bacterial plasmids, in the current study, were cured by exposing the cells to ethidium bromide. The plasmid cured isolate (isolate 51: *Escherichia coli*) lost the biocide resistance which was manifested by an increase in the inhibition zone diameters detected for all tested biocides. The increase in the susceptibility to biocides proved that the genes responsible for this resistant phenotype were plasmid-mediated rather than chromosomal-mediated (Russell, 1997).

Several studies have demonstrated that improved hygiene behaviors, particularly hand washing, are effective in reducing the incidence of certain infections (White et al., 2003; Kak, 2007). Findings underscore the importance of compulsory instruction in hand washing and sanitizing techniques as well as uniform distribution and access policies. It is accordingly recommended a two-part hand hygiene policy in public elementary schools: (1) Schools should ensure that all common areas are well-stocked with hand sanitizer and that all bathrooms are well stocked with hand washing materials throughout the school day. (2) Schools should provide a short hand hygiene lesson for students at the beginning of each academic year, as well as refresher lessons throughout the year (Lau et al., 2012).

The current microbiological sampling, while validated and reproducible, assessed only quantitative bacterial growth on a subset of environmental surfaces. Although the surfaces cultured represented a standardized set of commonly-touch items present across the school, other surfaces remained untested. The change of the location of environmental sampling would have altered the obtained data. Further study into environmental

reservoirs of infectious diseases may delineate the importance of surface contamination and define the relative impact of hygiene interventions in this important setting. The development of hygiene programs standardize the way the chemical agents should be handled (Penna et al., 2001).

In conclusion, the results of the current investigation led to the suggestion that official schools are highly contaminated with respect to private schools. Clorox® and Dettol® were the most effective biocides against the bacterial contaminants. Upon successful plasmid curing, the loss of resistance in previously resistant isolates proved that the genes responsible for this biocide resistant phenotype were plasmid-mediated rather than chromosomal-mediated. This emergence of bacterial resistance creates a challenge to school infection control and environmental decontamination protocols, and can have a serious impact on human health as well as economic consequences. For that reason, the scientific community must assess the importance of biocides and weigh the risks of their use against the benefits they provide. The development of hygiene programs standardize the way for chemical agents handling. It is essential to adequate control the concentration of diluted products, the contact time of the product, and the cleanliness of the surface prior to disinfection **(**Penna et al., 2001). In addition, the hardware aspects, such as the physical infrastructure, sanitation facilities at schools and the availability of safe water should be evaluated. Eventually, it is recommended to provide schools with knowledge on hygienic methods followed by their continued practices. Further studies should be conducted to determine the optimal intervention to reduce microbial contamination in the schools environment.

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