Effect of Cetrimonium Chloride on the Resistance Pattern of *Mycobacterium Tuberculosis* to Streptomycin

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Abstract

Household biocides have been used extensively all over the world, Kenya included. However, it has been recognized that their use may lead to failure of microorganisms to respond to pharmaceutical antimicrobials. One such biocide which has received little research attention is Cetrimonium Chloride, an antiseptic disinfectant which may be found in many household products such as shampoos and cosmetics. The purpose of this study was to investigate the impact of Cetrimonium Chloride on the development of resistance by Mycobacterium tuberculosis strains against therapeutic drugs designed for their control. Direct Drug Susceptibility Testing by Proportion Method was used. The dependent variable was number of colonies of Mycobacterium tuberculosis. The independent variables that were treated as between subjects factors were time of exposure and concentration of biocide. Findings indicated that the mean number of colonies decreased with time as well as concentration of biocide. All pairwise differences in mean number of colonies was statistically significant across time of exposure and concentration of biocide (p=.000). However, these main effects must be interpreted with caution because there was a statistically significant interaction effect between time of exposure and concentration of biocide (p=.000). The biocide had the greatest inhibitory or bactericidal effect at the highest level of concentration, regardless of time of exposure. The R^2 value was .791 implying that 79.1% of the variance in number of colonies was shared in common with the independent variables, inclusive of the interaction term. A dilution above 79.1% would mean that the colonies would start adapting by forming resistance genes.

Key words: Biocide, Cetrimonium Chloride, Mycobacterium tuberculosis, Resistance, Colonies, Concentration.

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INTRODUCTION

The result of misuse of antimicrobial drugs has over time been considered to be the main reason behind the adaptation of various micro-organisms to withstand their intended effects. This has been attributed to under-dosage, over-dosage or misdiagnosis of the patient by medical practitioners and subsequent administration of the wrong drugs. Most studies have been based on this precept.

The recognition that building irresponsiveness of microorganisms to pharmaceutical antimicrobials could lead to an apocalyptic phenomenon has led the world body on health-World Health Organization (WHO), to adopt policies and compel governments to implement measures in a bid to prevent such a scenario. For instance, in the year 1998 WHO adopted a resolution urging member states to take action against antimicrobial resistance; in 2001 a publication on the global strategy on containment of antimicrobial resistance was released. In addition to this, individual states and union states have also acted unilaterally or bilaterally to provide interim if not long term solutions. For instance, in 2009 the United States and the European Union jointly established the Transatlantic Task Force on Antimicrobial Resistance (TATFAR) which aimed at establishing a protocol for measuring antimicrobial use in hospitals (https://www.cdc.gov/drugresistance/tatfar/index.html). In 2021, TATFAR welcomed the United Kingdom, another global leader in the fight against antimicrobial resistance. Without doubt, this hints that, with regard to this context, the urgency to solve this problem has triggered international collaborative efforts in combating resultant mutagenic diseases. There is therefore need to further explore the resistance pattern of *Mycobacterium tuberculosis* to streptomycin.

LITERATURE REVIEW

Until about 50 years ago, tuberculosis (TB) was considered virtually incurable. The discovery of several antibiotics during that period heralded a new age of anti TB chemotherapy WHO (1997). A few years later it became apparent that the use of these drugs led to rapid drug resistance and subsequent treatment failures. These were important because with streptomycin *monotherapy (one drug treatment)*, resistant mutants began to appear within a few months endangering the success of antibiotic therapy. However, it was soon demonstrated that this problem could be overcome with the combination of two or three drugs (Daniel & Bates, 1994).

A number of anti-TB agents have been discovered including para-aminosalicyclic acid (1946), INH (1952), pyrazinamide and cycloserine (1952), ethambutol (1962) and rifampicin (1963). In the late 1980s it was evident that TB appeared to be a renewed threat that was much more pronounced in HIV patients, as an opportunistic disease, than any other (Daniel & Bates, 1994). Chances are that only one out of ten immune-competent people infected with *M. tuberculosis* fall sick in their lifetimes with active TB, but among those with HIV, one in ten per year develop active TB, while one in two or three tuberculin test positive AIDS patients develop active TB (Paolo & Nosanchuk, 2004). Exposure to biocides may be leading to increased antibiotic resistance, but this has not yet been proven to a large extent in clinical settings (IFH, 2003). Further research is needed to establish a correlation between biocide exposure and development of antibiotic resistance. This study focuses on Cetrimonium chloride, a typical household biocide in Kenya, as a possible contributor to antibiotic resistance. The nature of the biocide is described below.

Nature of Cetrimonium chloride

Household biocides, packaged as cleaning agents, are used extensively in urban and semi-urban areas in Kenya. One such biocide is Cetrimonium chloride, an antiseptic disinfectant which may be found in many household products such as shampoos and cosmetics. It is contained in products used for cleaning lavatories in combination with other ingredients such as hydrochloric acid. The IUPAC name is hexadecyl-trimethylammonium chloride and is represented by the molecular formula $C_{19}H_{42}ClN$ with molar mass of 320.00g/mol (https://www.google.com/search?q=cetrimonium+chloride+formula&rlz).

The Cetrimonium cation is an antimicrobial agent against bacteria and fungi. It has properties of disrupting micro-organisms' cell processes and surfactants. It is a quaternary ammonium compound.

Purpose of the Study

The purpose of the study was to investigate the impact of Cetrimonium chloride on the continuous development of resistance by *Mycobacterium tuberculosis* strains against therapeutic drugs designed for their control.

Specific Objectives

The objectives of the study were to:

i. Determine whether resistant mutants against streptomycin develop when exposed to conditions below the minimum bactericidal concentration of the biocide.

ii. Establish the relationship between use of Cetrimonium Chloride as a household biocide and antibiotic resistance to MDR/XDR tuberculosis.

Null Hypotheses of the Study

The following null hypotheses were tested to address the above objectives:

H_{01}: Resistant mutants of *Mycobacterium tuberculosis* against streptomycin do not develop as a result of use (misuse) of biocide product containing Cetrimonium chloride.

H_{02}: There is no relationship between use (misuse) of regular household biocide, Cetrimonium chloride, and mutation of *Mycobacterium tuberculosis* against streptomycin.

Significance of the Study

The findings of the study may be useful to government/policy makers, researchers and scholars, pharmaceutical industry, and the health sector in general.

Theoretical Framework

This study was guided by the Theory of Evolution. This theory states that "*modern organisms are descendants of ancient organisms and that modifications accumulated over time explain the apparent changes and differences among modern forms of life.*"

In 1859, Van dyke explained the principle of natural selection as the non-random process by which biological traits become either more or less in a population as a function of differential reproduction of their bearers. Natural selection is widely considered as the primary explanation behind adaptive evolution as it explains how species could change over time by adapting to environmental changes.

If the traits that give the organisms a reproductive advantage are also heritable, then a higher proportion is likely to be observed in subsequent generations.

Through mutations, natural populations of bacteria can exhibit variations in their genomes. If the populations are exposed to adverse environment for instance, with active antimicrobial agent, most die quickly but some have mutations that render them tolerant hence may survive especially if exposure time is short and the concentration of the antimicrobial agent is below the minimum.

MATERIALS AND METHOD

Direct Drug Susceptibility Testing (DST) by Proportion Method (PM) was used following the approach by Musa *et al.* (2005). Lowenstein Jensen (LJ) medium slants were incorporated, with streptomycin at critical concentration and drug free slants used as controls. The requirements included Cetrimonium Chloride, Mycobacterium tuberculosis isolates (from seven samples), Incubator (37°C), and wire loop for inoculation.

The Mycobacterium tuberculosis isolates were exposed to low concentrations of Cetrimonium Chloride. Lowenstein Jensen medium tubes were grouped into four sets according to the time in which the samples were exposed i.e. 48hours, 72 hours, 96 hours and 120 hrs. Each set had five tubes with at least one of the four tubes having antibiotic streptomycin as drug free control.

Part of the isolate suspension was diluted 1:100, and 200 µl of the dilution was inoculated on tubes of LJ medium one without any antibiotics (drug free control) and 200 µl of the undiluted suspension was inoculated, with respect to time of exposure, into the 112 LJ medium tubes with antibiotic streptomycin incorporated at critical concentration.

All tubes were incubated at 37°C immediately after inoculation. Preliminary results were reported as early as after 20 days. Final susceptibility results were reported only after 40 days following the standard procedure. An isolate was reported as resistant if the number of colonies growing on the antibiotic containing medium was 1% or more of the number of colonies developing on the drug-free control.

In this study, the dependent variable was number of colonies of Mycobacterium tuberculosis. The independent variables that were treated as between subjects factors were time of exposure and concentration of biocide. Time of exposure had four levels (Level 1=48 hours, Level 2=72 hours, Level 3=96 hours and Level 4=120 hours). Similarly, concentration of biocide had four levels (Level 1=0.15% v/v, Level 2=0.20% v/v, Level 3=0.25% v/v and Level 4=0.30% v/v). The design was balanced for each of the between subject factors, with 28 cases at each level. The selection of the four levels of concentration was in consideration of the fact that levels beneath minimum inhibitory concentration would create stressful conditions to the bacteria, and therefore induce intrinsic or extrinsic responses that would allow survival of future generations against higher concentration of the same biocide or different antimicrobial but with similar mechanisms of action. Below the minimum inhibitory concentrations however, the specific levels of concentration were selected arbitrarily provided that they were in increasing order and the values were not distant from each other which served to show trends.

Data were subjected to statistical treatment by determination of marginal means of colonies as influenced individually by the concentration and time and a visual graphic presentation was then done using bar charts. Tukey's multivariate test was then used compare the means i.e. Pairwise comparisons for mean number of colonies across time, and pairwise comparisons for mean number

of colonies across concentration of biocide. The interaction effect of concentration and time on mean number of colonies was determined i.e. Tests of between-subjects effects which was presented on line graph.

RESULTS

Culture of M. tuberculosis

The culture of *M. tuberculosis* on egg nutrient substrate after exactly three weeks of incubation at 37°C yielded rough, yellow to brown, cauliflower like colonies similar to what is shown in Figure 1.

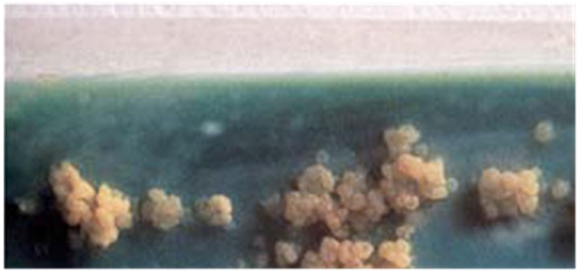


Fig. 1: Growth of *Mycobacterium tuberculosis* colonies on Lowenstein Jensen medium after three weeks incubation.

Mean Number of Colonies across Time of Exposure and Concentration of Biocide

Table 1 shows estimates of marginal means for number of colonies across time of exposure and Table 2 shows estimates of marginal means for number of colonies across concentration of biocide. The information is presented using bar charts in Figures 2 and 3, respectively. The charts indicate that the mean number of colonies decreased with time as well as concentration of biocide.

Table 1 : Marginal means for number of colonies across time of exposure

Time of exposure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
1=48 hours	8.214	.454	7.313	9.116
2=72 hours	6.500	.454	5.598	7.402
3=96 hours	4.214	.454	3.313	5.116
4=120 hours	2.786	.454	1.884	3.687

Table 2: Marginal means for number of colonies across concentration of biocide

Concentration of biocide	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
1=0.15% v/v	9.464	.454	8.563	10.366
2=0.20% v/v	7.071	.454	6.170	7.973
3=0.25% v/v	4.607	.454	3.706	5.509
4=0.30% v/v	.571	.454	-.330	1.473

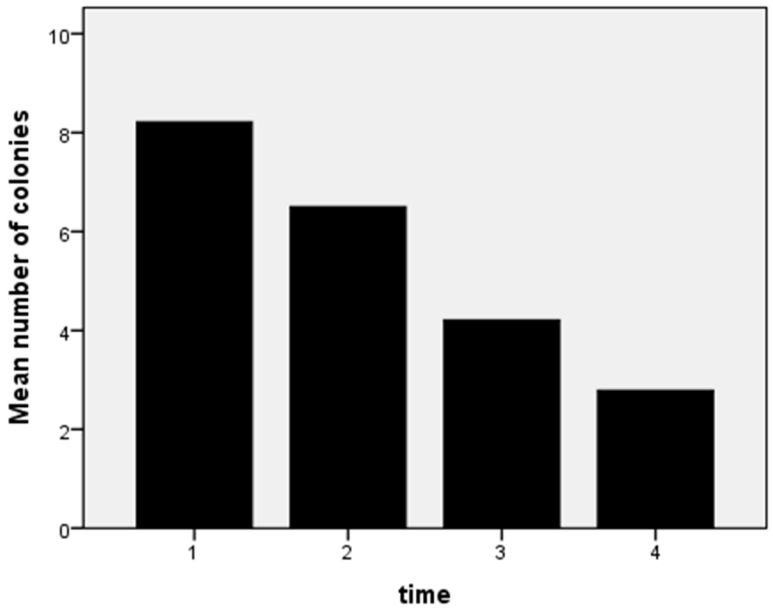


Figure 2: Mean number of colonies across time of exposure

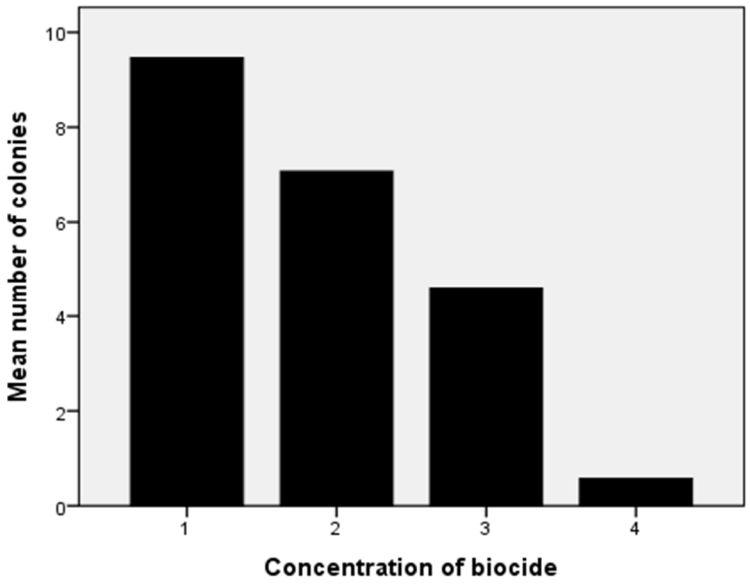


Figure 3: Mean number of colonies across concentration of biocide

In order to determine if the difference in mean number of colonies was statistically significant across time of exposure and concentration of biocide at α=.05, pairwise comparisons were conducted using Tukey's Least Significant Difference and the results are given in Table 3 and Table 4. The results indicate that all pairwise differences in mean number of colonies was statistically significant across time of exposure and concentration of biocide (p=.000).

Table 3: Pairwise comparisons for mean number of colonies across time

(I) time	(J) time	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
1	2	1.714*	.642	.009	.439	2.989
	3	4.000*	.642	.000	2.725	5.275
	4	5.429*	.642	.000	4.153	6.704
2	1	-1.714*	.642	.009	-2.989	-.439
	3	2.286*	.642	.001	1.011	3.561
	4	3.714*	.642	.000	2.439	4.989
3	1	-4.000*	.642	.000	-5.275	-2.725
	2	-2.286*	.642	.001	-3.561	-1.011
	4	1.429*	.642	.028	.153	2.704
4	1	-5.429*	.642	.000	-6.704	-4.153
	2	-3.714*	.642	.000	-4.989	-2.439
	3	-1.429*	.642	.028	-2.704	-.153

*. The mean difference is significant at the .05 level.
a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Table 4: Pairwise comparisons for mean number of colonies across concentration of biocide

(I) conc	(J) conc	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
1	2	2.393*	.642	.000	1.118	3.668
	3	4.857*	.642	.000	3.582	6.132
	4	8.893*	.642	.000	7.618	10.168
2	1	-2.393*	.642	.000	-3.668	-1.118
	3	2.464*	.642	.000	1.189	3.739
	4	6.500*	.642	.000	5.225	7.775
3	1	-4.857*	.642	.000	-6.132	-3.582
	2	-2.464*	.642	.000	-3.739	-1.189
	4	4.036*	.642	.000	2.761	5.311
4	1	-8.893*	.642	.000	-10.168	-7.618
	2	-6.500*	.642	.000	-7.775	-5.225
	3	-4.036*	.642	.000	-5.311	-2.761

Interaction Effect of Concentration of Biocide by Time of Exposure

Table 5 shows tests of between-subjects factors. As explained earlier, the main effects of time of exposure and concentration of biocide was statistically significant (p=.000). Whereas the main effects of time of exposure and concentration of biocide on number of colonies were statistically significant at α=.05, the finding must be treated with caution because there was a statistically significant interaction effect between the two as shown in Table 5 with p=.000.

Table 5: Tests of between-subjects effects

Source	Type III Sum of Squares	*df*	Mean Square	*F*	Sig.
Corrected Model	2100.857[a]	15	140.057	24.245	.000
Intercept	3300.571	1	3300.571	571.351	.000
Time	486.286	3	162.095	28.060	.000
Concentration	1211.071	3	403.690	69.882	.000
time * concentration	403.500	9	44.833	7.761	.000
Error	554.571	96	5.777		
Total	5956.000	112			
Corrected Total	2655.429	111			

a. R Squared = .791 (Adjusted R Squared = .759)

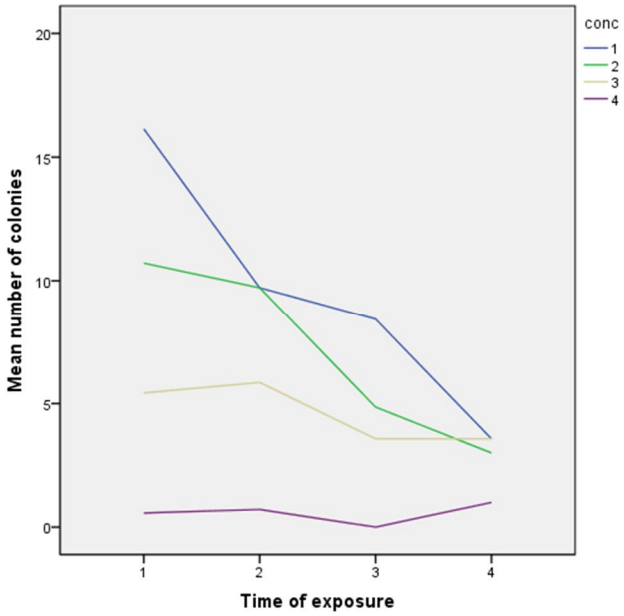
Figure 4 shows a line graph for the mean number of colonies against time of exposure for different levels of concentration of biocide. The Figure indicates that the estimated marginal means of colonies at the fourth level of concentration of biocide (0.30%v/v) was more or less constant across the four levels of time of exposure. It is at this level of concentration that the lowest estimated marginal means of colonies was realized, and the count was near zero. This suggests that the biocide had the greatest inhibitory or bactericidal effect at the highest level of concentration, regardless of time of exposure.

This level is more or less closer to the minimal bactericidal concentration of the biocide. In other words, this is close to the minimum threshold concentration that needs to be used to realize any benefits from the biocide.

Fig. 4: Mean number of colonies against time of exposure for different levels of concentration of biocide

Key: conc=Concentration of biocide.

The R^2 value was .791, which means that 79.1% of the variance in number of colonies was shared



in common with the independent variables, inclusive of the interaction term. A dilution above 79.1% would mean that the colonies would start adapting by forming resistance genes. Indeed, it is this interaction effect that remains important over and above the main effects. This is an important finding which is lacking in most of the previous research.

The type of concentration by time interaction for the remaining three levels of concentration was disordinal, which means that at concentration of 0.15% v/v, 0.20% v/v and 0.25% v/v, differences in number of colonies at lower levels of time of exposure was much larger than differences at 0.30% v/v. As a matter of fact, the differences remained minimal at 120 hours of exposure. This means that at this level, regardless of concentration level of the biocide, nearly the same number of colonies was realized. It is worth noting that it was at this level that the strongest bactericidal effect occurred.

DISCUSSION

The growth of *Mycobacterium tuberculosis* colonies on Lowenstein Jensen medium incorporated with streptomycin at critical concentration was an indication that mutants were generated as a result of the treatment with Cetrimonium Chloride. The treatment was done to simulate conditions under natural circumstances which would otherwise be as a direct consequence of misuse of the biocide in question similar to the approach used by Huet *et al.,* (2008).

The fact that the findings were significant at α=.05 serves as a basis to conclude that there is a relationship between use (misuse) of regular household biocide, Cetrimonium Chloride, and mutation of *Mycobacterium tuberculosis* against streptomycin. Therefore any misuse that leads to optimal conditions similar to those effected by concentration level three and four, both at time three and four, can confidently be considered to result to streptomycin resistance.

The shorter the time of exposure the lower the mean number of colonies and the higher the concentration the smaller the number of colonies. This behavior was very much anticipated. However, of greater importance to the study was the interaction effect of both the biocide concentration and time. The relationship between low concentration biocide and bacteriophage causes a surge in resistance. These cannot be conclusively explained by the interaction effect displayed by concentration levels 1 and 2 because either way, the number of colonies tends towards zero and given enough time there is a possibility that they may all die. As noted by Huet *et al.*, (2008), this could have been because they were at critical concentration level and almost at critical time in the reaction system. However, the third and fourth level of concentration shows that after the general drop in the number of colonies, there was a rise. This important finding could be an indication that the potency of the antibiotic streptomycin had been shrugged off by the *Mycobacterium tuberculosis* as a result of the previous biocide concentration and time interaction. This tends to confirm the postulate that suggests that the use of biocide below critical concentration at atomic level results to the conferment of *M. Tubercobacilli*-Crossresistance, through negative evolution as suggested by Russell and Mcdonnell (2000), Walsh *et al.,* (2003), Malek *et al.,* (2002), Langsrud *et al*. (2003), Maillard (2007) and Tattawasart *et al.* (1999).

The findings in this study are in support of the theory of negative evolution as postulated by Tortora, Funke and Case (2004). The theory suggests that negative evolution occurs in a Mycobacterium *tubercobacilli* through jumping genes called transposons and retro-transposons. Also consistent with the theory is the finding of Ugler *et al.* (2009) that streptomycin displays antimicrobial activity by inhibition of protein synthesis which is the ultimate end cycle of the negative evolution. Findings in this study are similar to those of Fernanda *et al*. (2013) who found that the minimum inhibitory concentration for Streptomycin in the presence of efflux pump inhibitors and the presence of rpsL, rrs and gid B genes provide evidence for the possible participation of efflux pumps in low level Streptomycin resistance.

In summary, any misuse that leads to conditions (i.e. optimal conditions) similar to those effected by concentration levels of 0.25% v/v and 0.30% v/v, both after 96 hours and 120 hours, can confidently be considered to result to streptomycin resistance at atomic level.

CONCLUSION

Resistance of *Mycobacterium tuberculosis* against streptomycin is due to exposure of the isolates to biocide with the active ingredient Cetrimonium chloride (i.e. selective/induced resistance). It was established that there was a relationship between misuse (use) of the biocide and the overall increased resistance against streptomycin by *Mycobacterium tuberculosis.* The resistance pattern observed in this study gives evidence that resistant mutants are generated from exposure to Cetrimonium chloride-based biocides. This should be of high concern as it raises pertinent questions on what its continual unregulated use would have, both currently, and in future, on the treatability of tuberculosis. A continuation of the trend is likely to lead to multiple drug resistance and indeed even worse drug resistant TB.

RECOMMENDATIONS

The following are recommendations from the study findings:

i. Deliberate efforts should be put in place by relevant authorities to monitor the use of Cetrimonium chloride so that conditions that are optimal for development of resistant mutants is eliminated during either house hold or other application. More specifically, the dilution of the biocide using water should not go above 79.1%, otherwise genes in the specific regions of *Mycobacterium tuberculosis* genome would mutate and this is likely to render streptomycin inactive.

ii. The use of antibiotics that cross-react should be halted and only those that have been proven not to cross-react should be administered in therapies.

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REFERENCES

Anders, A., James, B., Jong, D. W., *et al.* (2009). *Assessment of Antibiotic Resistance Effect of Biocides.* Brussels: European Commission.

Bennett, P.N. & Brown, M. J. (2003). *Clinical Pharmacology*, 9th ed. London: Churchil Livingstone.

Centers for Disease Control and Prevention. *Transatlantic Taskforce on Antimicrobial Resistance (TATFAR)*. Retrieved from **https://www.cdc.gov/drugresistance/tatfar/index.html** on September 1st, 2022.

Central TB Division et al., (2009). *National TB Training Manual for Mycobacterium tuberculosis culture and drug susceptibility testing.* New Delhi.

Cetrimonium chloride/*Formula*. Retrieved from https://www.google.com/search?q=cetrimonium+chloride+formula&rlz on August 17th 2022.

EARSS (European Antimicrobial Resistance Surveillance System). *Annual Report 2005*. Available from: https://www.rivm.nl/bibliotheek/rapporten/210624001.pdf (accessed September 1st, 2022).

Evans, A., (2008). Multi drug resistant TB: A Challenge in Management of Tuberculosis. *African Journal of Health Science, 15,* 6-13.

Fernanda, S., *et al.*, (2008). Identification of Mutations related to Streptomycin resistance in Clinical isolates of Mycobacterium tuberculosis and possible involvement of Efflux mechanism. *American Society for Microbiology*, 2947-2949

Fraise, A. P. (2002). Biocide abuse and antimicrobial resistance-a cause for concern? *Journal of Antimicrobial Chemotherapy, 49,* 11-12.

Harbarth, S. & Samore, M. H. (2005). Antimicrobial resistance determinants and future control. *Emerging Infectious Diseases, 11*:794-801.

Huet, A. A. *et al.* (2008). Multidrug efflux pump overexpression in *Staphylococcus aureus* after single and multiple *in vitro* exposures to biocides and dyes. *Microbiology, 154*:3144-53.

IFH (International Scientific Forum for Home Hygiene): Biocide usage and antimicrobial resistance in home settings: An update. October 2003.

Iruk, O. N. & Anibal, S. (1999). *Antibiotic Resistance in Africa; Discerning the Enemy and Plotting a Defense*. Lancaster: Haverford College.

Karatzas, K. A. *et al.* (2007). Treatment of Salmonella enterica serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. *J Antimicrob Chemother; 60*:947-55.

Poole, K. (2007). *Efflux pumps as antimicrobial resistance mechanisms*. *Ann Med; 39*:162-76.

Richard, J. L., & Morris L. M., (1981). *An introduction to mathematical statistics and its applications.* N.J.: Prentice-Hall, Englewood Cliffs.

Russell, A. D. (2003). Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical environmental situations. *Lancet Infect* Dis; 3:794-803.

Paolo, W. F. & Nosanchuk, J. D. (2004). Tuberculosis in New York city: Recent lessons and a look ahead. *Lancet Infectious Diseases, 4*(5), 287-293. Retrieved from https://doi.org/10.1016/S1473-3099(04)01004-7 on August 1st, 2022.

Tortora, G. J., Funke, B. R. & Case, C. L. (2004). *Microbiology: An introduction*. San Francisco: Pearson Education Inc.

Ugler, M. *et al.*, (2009). Investigation of rpsL and rrs gene mutations in Streptomycin resistant Mycobacterim tuberculosis. *PubMed*: 115-120.