Study on the antibacterial activity of liquid hand-wash containing triclosan against clinical samples

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by
Fuad Mustafa
Student ID: 13336010
October, 2017

Biotechnology Program
Department of Mathematics and Natural Sciences

BRAC University

For my beloved parents and brother

Declaration

I hereby declare that this thesis entitled **"Study on the antibacterial activity of liquid hand-wash containing triclosan against clinical samples"** is submitted by me, Fuad Mustafa, to the Department of Mathematics and Natural Sciences under the supervision and guidance of Lecturer Nazneen Jahan and Troshporsha Tasnim Khan of Department of Mathematics and Natural Sciences, BRAC University. I also declare that the thesis work presented here is original, and has not been submitted elsewhere for any degree or diploma.

Candidate
Fuad Mustafa
ID: 13336010

Certified by
Nazneen Jahan
Supervisor
Lecturer,
Microbiology Program
Department of Mathematics and Natural Sciences
BRAC University

Certified by
Troshporsha Tasnim Khan
Co Supervisor
Lecturer,
Microbiology Program
Department of Mathematics and Natural Sciences
BRAC University

Acknowledgement

I would like to begin by thanking the Almighty for guiding me and giving me the patience I needed to complete this thesis successfully.

I express my sincere gratitude to **Professor A F M Yusuf Haider,** Chairperson, Department of Mathematics and Natural Sciences, BRAC University, for his valuable supervision and cooperation. I express my heartfelt gratitude to **Professor Naiyyum Choudhury**, Former Co-ordinatior, Biotechnology and Microbiology, Department of Mathematics and Natural Sciences and Chairman, Bangladesh Atomic Energy Regulatory Authority.

I am much obliged to my thesis supervisor, **Lecturer Nazneen Jahan** and co-supervisor, **Lecturer, Troshporsha Tasnim Khan**, Department of Mathematics and Natural Sciences, BRAC University, for their continuous support, guidance and encouragement throughout the duration of my thesis. Without their expert suggestions and devoted involvement, this thesis would not have taken shape. I am eternally grateful to them for believing in me and for staying with me through all the hurdles.

My regards and gratitude go to **Professor Dr. Aparna Islam, Assisant Professor Jebennesa Chowdhury, Senior Lecturer Romana Siddique, Lecturer Zubaida Marufee Islam Lecturer Shamira Tabrejee and Lecturer Kashmery Khan,** Department of Mathematics and Natural Sciences, BRAC University, for all their support and encouragement throughout the duration of my undergraduate degree.

Without the assistance and encouragement of my family, as well as Noshin Sharmili Hridi, Thariqul Islam Rocky, Mir Shohayeb Rabbi, Nadia Binte Obaid, Arko Roy, Fahim Sarwar, Syed Akib Hossain, Promi Tahsin, Rabeya Tafsire Rudhy, Salman Khan Promon, Asma Binte Afzal, Nahreen Mirza and Moutushi Chowdhury, I would not have been able to complete this project. I sincerely thank every single one of them for being such huge sources of inspiration.

Fuad Mustafa

October, 2017

Table of contents:

List of tables:

List of figures:

List of Abbreviations:

Abbreviation	***Elaboration***
APHA	American Public Health Association
DCDD	Dichlorodibenzo-4-dioxin
DCP	Dichlorophenol
ENR	Enoyl-acyl carrier protein reductase
EPA	Environmental Protection Agency
FabI	Fatty acid biosynthesis
FD & C	Federal Food, Drug & Cosmetic Act
FDA	Food and Drug Administration
GRAE	Generally Recognized as Effective
GRAS	Generally Recognized as Safe
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
OprM	Outer Membrane Protein
ORD	Office of Research & Development
OTC	Over the Counter
PACD	Photo Allergic Contact Dermatitis
PBP	Penicillin Binding Protein
PCMX	Para-chloro-meta-xylenol
RED	Re-registration Eligibility Decision
TFM	Tentative Final Monograph
USA	United States of America
WHO	World Health Organization

Abstract:

In recent times the use of antibacterial hand washes has been heavily promoted to a health-conscious public. The purpose of this study was to investigate *in vitro* antimicrobial activity of liquid hand soap containing triclosan against microorganisms. Eight clinical isolates were used in this studyand they were *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Salmonella typhi, Proteus vulgaris, Shigella flexneri, Bacillus subtilis* and *Staphylococcus aureus.* Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was determined for the microorganisms through Broth Microdilution method. Kirby-Bauer disc diffusion test of the same organisms was done using the following antibiotics: Erythromycin (15μg), Chloramphenicol (30 μg), Ciprofloxacin (5μg), Gentamicin (10 μg), Kanamycin (10 μg), Nalidixic acid (30 μg), Penicillin (10μg), Streptomycin (10μg), Tetracycline (30 μg). In this study, *Salmonella typhi* showed the highest MIC value (3.0 ml of stock solution) and *Bacillus subtilis, Staphylococcus aureus* had shown the lowest MIC value (0.6 ml of stock solution). Besides, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris, Shigella flexneri* had shown the values 2.6 ml, 2.4 ml, 2.0 ml, 1.0 ml, 0.8 ml of stock solution respectively. All of the clinical isolates taken for the study had shown resistant characteristics against at least six tested antibiotics and three isolates were resistant to all the nine antibiotics. It is possible that antibacterial liquid hand-washes have the antibacterial agents (e.g. Triclosan) that can either kill or inhibit the bacterial cells. It might be possible that some bacterial strain can develop resistant properties which leads to their survival even at higher concentration of soaps and antibiotics.

Chapter 1: Introduction

1.0 Introduction:

Cleansing agents have been used around us for a long time and among them soap, liquid hand-wash, detergent, etc. are noteworthy. Antibacterial soaps have been used as personal hygiene for generations. Bacteria are very sundry and diverse and can be found in water, soil, sewage, on human body and are of great importance with reference to health (Johnson et al., 2002). In the year 1961, the U.S Public Health Service Recommendation mentioned that personnel clean and wash their hands with soap and water for one to two minutes before and after client contact. The antibacterial soaps can clean and remove 65% to 85% bacteria from human skin (Osborne and Grube, 1982). Besides it should be noted that fats and oils are general ingredients of soaps but there are some detergent additives which enhance and improve the antibacterial activities of soaps (Friedman and Wolf, 1996).

Hand washing is very important and crucial when it is related to health care workers because of possible and probable cross contaminating of bacteria that may be pathogenic or opportunistic (Richards et al., 1999). Hygiene of hands and prevention of infection through the use of antibacterial liquid hand-wash has been well recognized. There are many and a large number of chemical compounds that have the potential to inhibit the growth, contamination and metabolism of microorganisms or kill them. The quantity and number of chemicals are vast and probably at least 10000 and among them 1000 chemicals are generally and commonly used in hospitals and homes. The important and significant groups of chemicals that help to destroy microorganisms are hydrogen, phenols, soaps, detergents, ammonia compounds, chlorine, alcohols, heavy metals, acids and certain compounds are available around us. Antisepsis, sanitization, disinfection, decontamination, sterilization and so on are a few terms that tells the process of cleaning by any cleansing agents. Various and several cleansing agents are available in the market that are found in various forms and in different formulations. Trichlorocarbanilide, triclosan and P-chloro-in-xylenol (PCMX/ Chloroxylenol) are the mostly used antibacterial in medicated soaps. Actually, these are generally only contained at preservation level unless the product is properly marked as antibacterial, antiseptic or germicidal (Larson et al., 1989).

Washing, scrubbing our body or hands with soaps is the first of defense against bacteria and other pathogens that can affect us with flu, skin infection and even deadly communicable diseases (Kimel, 1996). Usually, most of the people believe that an

antimicrobial portion of soaps is effective at preventing communicable diseases. It is to be noted that now many researchers mention that high use of antimicrobial chemicals can have the reverse effect of spreading diseases and infections instead of preventing them (Poole, 2002). Antimicrobial resistance and rendering an individual more vulnerable to more microbial attacks like diseases or infections can result due to over utilization of antibacterial chemicals (White and McDermolt, 2001). High use of these agents can give rise to drug resistant microorganisms in the future.

1.01 Background:

Triclosan is a chemical compound which generally possess anti-bacterial properties and sometimes it also works on fungus and viruses. It is commonly used for destroying bacteria on skin, surfaces and it occasionally is used to protect the product against deterioration due to microbes. The use of triclosan has first started in U.S.A in the 1970s on soaps and the use of triclosan has been radically increased in the past few years. Antibacterial agents like triclosan and the by-products of antibacterial agents have been found all over the environment like surface water, soil, fish, tissue and human breast milk (Adolfsson et al., 2000). In the year 2009, the American Public Health Association (APHA) suggested that it would sanction the exclusion of triclosan for household and non-medical uses. Triclosan is a chlorinated bisphenol or a phenylether which has a vast-range of antimicrobial action and it is categorized as a Class III drug by the FDA and class-III drugs are compounds with high solubility and low permeability (Courtney and Moore).Its actual chemical name is 2,4,4′-trichloro-2′-hydroxy-diphenyl ether. Triclosan is produced and manufactured by Ciba Specialty Chemical Products by their commercial names Irgasan® and Irgacare® in U.S.A. Triclosan is also produced in China, India, Netherlands, Switzerland, South Korea and so on (Menoutis and Parisi-Menoutis). It has a faint aromatic, phenolic scent as it is a chlorinated aromatic compound. Triclosan can come in either ether or phenol form though the phenol forms are more popularly used as they have antibacterial properties.

1.02 Antibacterial liquid soap:

Antibacterial soap is a kind of cleaning product which consist of chemical ingredients that purportedly assist in killing bacteria (FDA, 2016). Hand liquid soap is prepared to wash and clean hands. Liquid hand soap is the best-selling and most widely used in detergent products groups. Liquid hand soaps may have diverse features depending on content. There are distinctions, such as for children, opaque, with glycerine, with antibacterial,

transparents and etc. Liquid hand soap contains anionic surfactant, cocamide, coco betaine as amphoteric surfactant, opaque agent as making opaque, dye, fragrance, glycerine for prevention hand outside conditions and etc. Studies have revealed that liquid soaps contain antimicrobial active ingredients which take away more bacteria as compared to plain soap (Aiello, 2008). Antibacterial soaps (sometimes called antimicrobial or antiseptic soaps) contain certain chemicals not found in plain soaps. Those ingredients are added to many consumer products with the intent of reducing or preventing bacterial infection.

1.03 Target organisms:

Triclosan has a large range of bactericidal activity that includes many types of microorganisms but not on all types of microorganisms like gram-negative and gram-positive non-sporulating bacteria, some fungi (Schweizer,2001), *Plasmodium falciparum* and *Toxoplasma gondii* (McLeod et al., 2001). It inhibits the growth of microorganisms i.e. bacteriostatic at lower concentration and it kills the bacteria at higher concentration i.e. bactericidal. There are many microorganisms that are very sensitive to triclosan like *Staphylococci,* some *Streptococci, E. coli,* some mycobacteria and *Proteus* spp. Besides methicillin-resistant *Staphylococcus aureus* (MRSA) strains are also highly sensitive to triclosan (Al-Doori et al.,2003). Patients bathing with 2% triclosan is a potential regimen for decolonization of patients whose skin is carrying MRSA (Tuffnell et al.,1987). *Pseudomonas aeruginosa* is highly resistant to triclosan and enterococci species like *Klebsiella pneumoniae, Escherichia coli* are usually less susceptible than staphylococci (Russel, 2003). Recent work with *Salmonella* has shown that development as a biofilm provides increased protection against the action of triclosan (Tabak et al., 2007). The microorganisms which are targeted:

1. ***Pseudomonas aeruginosa*:** *Pseudomonas aeruginosa* is a gram-negative, rod shaped bacterium. It is an opportunistic human pathogen and it is accelerated by its intrinsic resistance to antibiotics and disinfectants. It is a pervasive pathogen capable of infecting virtually all types of tissues (Lyczak et al., 2000).
2. ***Klebsiella pneumoniae*:** Bacteria of the genus *Klebsiella* are gram-negative, rod shaped, non-motile bacteria available almost everywhere in nature. These bacteria normally instigate human nosocomial infections. Hospital epidemics of multidrug resistant *Klebsiella* spp. have been increasing steadily over the past years (Podschun & Ullmann, 1998). The species *Klebsiella pneumoniae* is responsible for a

noteworthy portion of hospital acquired urinary tract infections, pneumonia, septicemia and soft tissue infection.

3. ***Escherichia coli:****Escherichia coli* are gram-negative microorganisms and also are commensal gut bacteria. These organisms can also cause urinary tract infections (Tortora et al., 2010). But there are some pathogenic strains which can sometimes produce enterotoxins that can create foodborne diseases and gastrointestinal infections.
4. ***Salmonella typhi****:* Typhoid fever remains a global health problem, resulting in more than 200,000 annual deaths, mostly children in developing countries (Pang et al., 1998) (Crump and Mintz, 2010). Unlike other *Salmonella enterica* such as *S.* Typhimurium or *S.* enteritidis, which are associated with gastroenteritis (i. e. "food poisoning") and can infect a variety of hosts, *S.* typhi is a special human pathogen (Parry et al., 2002). Furthermore, *S.* typhi can cause life-long infections in humans, most often by colonizing the gall bladder. It is believed that a combination of genome degradation and acquisition of new genetic information has conferred on *S.* typhi its unique pathogenic properties (Sabbagh et al., 2010). The isolation of multi drug resistant *S.* typhi has raised the worrisome possibility of the reemergence of untreatable typhoid fever (Mirza et al., 1996).
5. ***Shigella flexneri:****Shigella* species are the Gram-negative bacteria that cause shigellosis – a leading cause of bacillary dysentery in developing countries. Annually, 125 million cases of endemic shigellosis occur in Asia alone and children under 5 years of age are at the highest risk of illness and death (Bardhan et al., 2010). Among the four species of *Shigella*, *S. flexneri* is the primary cause of shigellosis in developing countries accounting up to 66% of all *Shigella* species infections (Gu B et al., 2012).
6. ***Proteus vulgaris:****Proteus vulgaris* is a rod-shaped Gram-negativechemoheterotroph bacterium. The size of individual cells varies from 0.4~0.6μm by 1.2~2.5μm. *P. vulgaris* possesses peritrichous flagella, making it actively motile. It inhabits the soil, polluted water, raw meat, gastrointestinal tracts of animals, and dust. In humans, *Proteus* species most commonly cause urinary tract infections, but can also produce severe abscesses; *P. mirabilis* produces 90 percent of cases, and is encountered in the community, but *P. vulgaris*is associated with nosocomial infection (O'Hara *et al.,* 2000).

7. ***Staphylococcus aureus****: Staphylococcus aureus* is both a commensal bacterium and a human pathogen. Approximately 30% of the human population is colonized with *S. aureus* (Wertheim et al., 2005). Simultaneously, it is a leading cause of bacteremia and infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections.
8. ***Bacillus subtilis:*** *Bacillus subtilis*, known also as the hay bacillus or grass bacillus, is a Gram-positive, catalase-positive bacterium, found in soil and the gastrointestinal tract of ruminants and humans. A member of the genus*Bacillus*, *B. subtilis* is rod-shaped, and can form a tough, protective endospore, allowing it to tolerate extreme environmental conditions. This species is commonly found in the upper layers of the soil, and evidence exists that *B. subtilis* is a normal gut commensal in humans. A 2009 study compared the density of spores found in soil (about 10^6 spores per gram) to that found in human feces (about 10^4 spores per gram). The number of spores found in the human gut was too high to be attributed solely to consumption through food contamination (Hong et al., 2009).

1.04 Mode of action of triclosan:

Triclosan performs by obstructing the active site of the enoyl-acyl carrier protein reductase enzyme (ENR) and it is a very important enzyme in fatty acid production in bacteria (Levy et.al, 1999). Triclosan inhibits the enzyme by obstructing the active site and fatty acid production of bacteria is hindered that is essential for making cell membrane and for reproduction. Triclosan has been considered as harmless to human because humans have no ENR enzyme. Proper neutralization of these antibacterial chemicals is often overlooked. Neutralization of triclosan is very difficult and as a result incomplete neutralization can overestimate the effectiveness of triclosan-containing products (McDonnell et al., 1998).

1.05 Antibiotic resistance in bacteria:

The discovery of antibiotics must surely rank as one of the greatest medical achievements of the twentieth century. Preliminary with the introduction into medical practice of the sulphonamides in the 1930s, penicillin and streptomycin in the 1940s, the broad spectrum bacteriostatic antibiotics during the 1950s, followed by bactericidal antibiotics in the 1960s, together with other important synthetic chemicals and highly specific narrow spectrum antibiotics during these years, one might have thought that the stage had been set for revolutionizing the treatment of bacterial diseases (Baldry, 1976). The usage of

antimicrobials for any type of infection in any dose over a period of time forces any microorganism to adapt or die and it is the surviving microbes which carry drug resistance genes and it may be transmitted to other strains within their own genus and species and across them even to another unrelated species. Bacterial resistance can be intrinsic or acquired. While intrinsic resistance is a naturally occurring trait arising from the biology of the organism e.g. vancomycin resistance in *Escherichia coli* (Hawkey, 1998), acquired resistance occurs when a bacterium which was previously sensitive to antibiotics grows resistance. This frequently happens by mutation or by gaining of new DNA (Tomasz and Munaz, 1995). Mutation is now recognized as the widespread mechanism of resistance development in bacteria especially in *Mycobacterium tuberculosis* (Musser, 1995). Resistance genes produced in the process are replicated and transferred to in-contact individuals via plasmids and transposons causing in the emergence of multi-drug resistant tuberculosis, which has now been identified in over 100 countries (Vachon, 1999). Besides mutation, bacteria have developed a diverse array of biochemical and genetic systems for confirming the evolution and propagation of antibiotic resistance (Hawkey, 1998). These include antibiotic modification such that it does not react with the target site such as occurs in b-lactamases which enzymatically cleave four-membered b-lactam ring, rendering the antibiotic inactive (Livermore, 1995). In few cases, antibiotic resistant bacteria may protect the target of antibiotic action by reducing antibiotic uptake or a quick efflux of it, as happens between b-lactam antibiotics and Gram negative bacteria ((Musser, 1995). Besides, the target antibiotic action may be altered thus rendering the antibiotic ineffective e.g. the resistance of enterococci to cephalosporins (Livermore, 1995). The final mechanism by which bacteria may protect themselves from antibiotic action is the production of an alternative target (usually an enzyme) that is resistant to inhibition by the antibiotic while the organisms continue to produce the original sensitive target. This allows bacteria to survive in the face of selection as the alternative enzyme bypasses the effect of the antibiotic. The best-known example is the alternative penicillin building protein (PBP2a) which is produced in addition to the normal penicillin building proteins by methicillin resistant *Staphyloccoccus aureus* (MRSA). It is however not uncommon to find a bacterium exhibiting more than one of these mechanisms (Hawkey, 1998).

1.06 Commercial antibiotics and antibiotic resistance:

Antibiotics are used widely to treat diseases in plants, animals and humans. After the discovery of penicillin in 1928, many other antibiotics have been discovered and

commercially produced. About 100,000 tons of antibiotics are manufactured annually worldwide, and their widespread use has profoundly affected bacterial life on earth. More and more strains are becoming resistant every day, and many have already become resistant to multiple drugs and chemotherapeutic agents-the phenomenon of multidrug resistance (Nikaido, 2010).

Antibiotics can be characterized according to their major mechanisms of action:

- Macrolides and tetracyclines are bacteriostatic and inhibit protein synthesis in bacteria [Example: Erythromycin, Azithromycin]
- β-lactams (and cephalosporins) interfere with bacterial cell wall synthesis, achieved by a competitive inhibition on PBP (penicillin binding proteins) [Example: Ampicillin, Cefuroxime, Ceftriaxone]
- Fluoroquinolones are synthetic antibiotics which belongs to the family of quinolones and they function by blocking bacterial DNA replication through inhibition of DNA gyrase [Example: Ciprofloxacin, Levofloxacin, Ofloxacin]
- Aminoglycosides also inhibit bacterial protein synthesis [Example: Amikacin, Gentamicin, Kanamycin]

Besides, other antibiotics perform by inhibiting metabolic pathways of bacteria and also by break-down of bacterial membrane structures (Tenover, 2006).

1.07 Bacterial resistance to triclosan:

Triclosan resistant bacteria can be produced clearly by isolation of resistant colonies within growth inhibition zones around a paper disc containing triclosan or by serial passage in increasing triclosan concentration (Russell,2003). In *E. coli* resistance, may occur for overproduction of the enzyme enoyl reductase or for changes in cellular permeability (Russell,2003). Actually, most resistant bacteria grow more slowly than sensitive bacteria and *E. coli* strains which are resistant to triclosan have boosted growth rates. The resistant strains can be able to tolerate and endure better when it is continuously exposed to triclosan and it may become more hard, strong, more resistant to triclosan.*Pseudomonas aeruginosa* is basically resistant to triclosan and this resistance could be for a non-susceptible enoyl reductase and both triclosan-susceptible and -non-susceptible enzymes have been found (Health, White and Rock, 2001), an external

membrane permeability barrier or a pumping of the drug from the cell interior to its exterior. MRSA strains may or may not demonstrate decreased sensitivity to triclosan (Bamber and Neal, 1999). All types of *S. aureus* strains which are having decreased sensitivity overproduced the enzyme FabI by three- to five-fold, and the most resistant strains had mutations in FabI (Fan et al., 2002).

1.08 Association between triclosan and antibiotic resistance:

A good number of current studies have elevated serious interests that triclosan and other related products may stimulate the emergence of bacteria resistant to antibiotics. One of the notable concern is that bacteria will become resistant to antibacterial products like triclosan and adapting the products unworkable to those who actually require them, such as people having compromised immune systems. Scientists also concern that because of triclosan‘s mode of action and target site in the bacteria is alike to antibiotics as well as bacteria that become resistant to triclosan will also become resistant to antibiotics. Triclosan does not truly trigger a mutation in the bacteria but it forms a situation where mutated bacteria that are resistant to triclosan are more expected to endure, survive and reproduce through killing of the bacteria. Triclosan's excess use could cause the development of cross-resistance to antibiotics and thus result in the development of strains of bacteria resistant to both triclosan and antibiotics was mentioned in article coauthored by Dr. Stuart Levy in 1998 (McMurry et al., 1998). The susceptibility of MRSA strains to triclosan has altered little over a 10 years period (Tuffnell et. al., 1987). Bacterial resistance to disinfectants in common is not a new occurrence and there are known examples of reduced susceptibility which is being termed over a century ago (Russel, 2004).Triclosan, of course, is of more recent vintage. Subsequently, it is essential to continue to observe whether reduced susceptibility to it and to antibiotics occurs. As resistance often builds in a step-wise fashion, it is practical to conserve use and continue investigation of susceptibility to both antibiotics and to biocides like triclosan.

1.09 Health effects of triclosan:

In 2003-2004, a data from National Health and Nutrition Examination Survey presented that triclosan is found in 75% of urine analyzed samples (Calafat et al., 2008). It is also found that triclosan is available in sewage sludge applied to agriculture, rivers, streams (Crinnion, 2010). Triclosan is relatively non-toxic for humans and other mammals in classical toxological terms. But there have been reports that due to exposure to triclosan,

contact dermatitis or skin irritation have occurred (Robertshaw and Leppard, 2007). Triclosan may cause photo-allergic contact dermatitis (PACD) and PACD happens when the portion of the skin is exposed to triclosan and also to sunlight. PACD is able to form an eczematous rash on the face, neck, the back of the hands and on the sun-exposed areas of the arms (Schena et al., 2008). Triclosan is found in three out of the five human milk samples in a Swedish study which directs us that triclosan is absorbed into human body in large amounts (Adolfsson et al.,2000). Triclosan can accumulate in fatty tissues because it is lipophilic. Triclosan interloping with the body‘s thyroid hormone metabolism was a concern which headed to a study that triclosan had a hypothermic effect, decreasing the body temperature, and producing a - nonspecific depressant outcome on the central nervous system of mice (Miller et al., 1983). In a very recent paper in *Environment International,* it is found that triclosan can hamper estrogen sulfotransferase in sheep placenta which is an enzyme that assists to metabolize the hormone and transport it to the developing fetus (James et al., 2010). The thought is that triclosan would be risky in pregnancy if adequate of it gets through to the placenta to affect the enzyme. Triclosan and its link t dioxins is an important issue. Dioxin is a carcinogen and can create many health problems like decreased fertility, altered sex hormones, miscarriage, weakening of immune system, birth defects, and cancer. Dioxin is a toxic group of compounds and only 17 are of public health concern out of 210 dioxin compounds (Van den Berg M et al., 2006). The two dioxin compounds-2,8-dichlorodibenzo-*p*-dioxin (2,8-DCDD) and 2,4-dichlorophenol (2,4-DCP) are synthesized by photochemical degradation of triclosan and when chemical by-products are exposed to UV radiation after the reaction of triclosan with chlorine water (Fiss, Rule and Vikesland, 2007).

1.1 Triclosan in environment:

Triclosan, and other antibacterial agents and their degradation byproducts are now found all-over the environment, with surface waters, soil, fish tissue, and human breast milk (Adolfsson et al., 2000). Swiss researchers obtained that three out of five samples of human breast milk had measurable concentrations of triclosan (at concentrations up to 30 μg/kg lipid weight) and over 95% of the uses of triclosan are in consumer products that are disposed of in residential drains. In a U.S., Geological Survey study of 95 different organic wastewater contaminants in U.S. streams, triclosan was one of the most frequently detected compounds, and in some of the highest concentrations. A study of triclosan in water bodies of Switzerland also found high concentrations of the chemical in several lakes and rivers

(Glasser, 2004). Triclosan was found in 57 percent of the 139 U.S. waterways that were assumed to be susceptible to agriculture or urban activities in a 1999-2000 study by the U.S. Geological Survey (Kolpin et al., 2002). Triclosan is detected in both wastewater and surface water. The source of surface-water may include wastewater treatment plant effluent, urban storm-water, rural storm-water, and agricultural runoff. Evidence is found that that up to 95 percent of triclosan is removed via the wastewater treatment plant process when domestic waste water is treated before discharge to surface waters (Samsøe and Petersen, 2003). Swiss researchers projected that 79 percent of the triclosan was removed through biological degradation while 15 percent adsorbed to the sludge and the rest 6 percent in the effluent has developed in a concentration of 42 ng/Liter (H. Singer et al., 2002). The transportation of triclosan to wastewater treatment plants happens when people wash hands with antibacterial soap, wash dishes with antibacterial soap, clean dirt with antibacterial products, shower with antibacterial soap or shampoo, use toothpaste containing antibacterial products, wash clothes with antibacterial products, etc. Triclosan may be moved into the water system through commercial or residential washing of apparatus with antibacterial soaps. The existence of triclosan may affect both the structure and the function of algal groups in stream ecosystems which are receiving treated wastewater effluent (B.A. Wilson et al., 2003). It may severely affect the natural nutrient capacity of the stream and the structure of the food web of the ecosystem of the streams.

1.11 Regulation of the use of triclosan:

Triclosan is coming under close inspection in this present period. In March of 2010, the European Union banned triclosan for any products that may come into contact with food, and in August of 2009 the Canadian Medical Association asked the Canadian government to ban triclosan use in household products under concerns of creating bacterial resistance and producing dangerous side products. And on September 2, 2016, the Food and Drug Administration (FDA) banned 19 chemicals and among them triclosan is mentioned (FDA, 2016). In the United States if an antimicrobial product is intended for use on the human body, it falls under the jurisdiction of the Food and Drug Administration.When a product makes a health-related issue like killing of germs, FDA termed it as a drug. The FDA standardizes drugs alike to the way that the EPA regulates pesticides by using a risk-benefit analysis based on data gathered from animal studies and human clinical trials. The manufacturer must verify that the drug is safe and operative in its proposed uses and that the aids of the drug compensate the risks; the drug ‘s proposed labeling is appropriate; and

the manufacturing methods used are able to maintain the drug ‘s quality, identity, strength, and purity. On the other hand, the FDA is only able to regulate cosmetics after products are released commercially. Neither cosmetic products nor cosmetic ingredients are examined or approved by the FDA before they are sold to the public. The FDA cannot order companies to do safety testing of their cosmetic products before marketing.

The EPA circulated in the Federal Register a petition which is filed by 82 public health and environmental groups, led by Beyond Pesticides and Food and Water Watch, to prohibit triclosan for non-medical use on December 8, 2010. On April, 2010 the FDA announced it is directing a scientific and regulatory review of triclosan in FDA-regulated products, with publication of results expected in spring 2011 (FDA, 2010). The agency also is collaborating with the EPA specifically to study the potential endocrine-disrupting effects of the compound (EPA, 2010). The US FDA began the drug review monograph process for "over-the-counter (OTC) topical antimicrobial products," including triclosan and triclocarban in 1974. In 1978 FDA circulated a tentative final monograph (TFM) for topical antimicrobial products. In the 1994, triclosan was effectively removed from the drug category which made it available for use in consumer products. In 2010, the Natural Resources Defense Council forced the FDA to review triclosan after suing them for their inaction.

The EPA decided a Re-registration Eligibility Decision (RED) document for triclosan in 2008. This RED document described the conclusions of EPA‘s wide-ranging review of the potential risks to human health and the environment resulting from the registered pesticidal uses of triclosan. In running the review for the RED, the EPA considered all available data on triclosan, including data on endocrine effects, developmental and reproductive toxicity, chronic toxicity, and carcinogenicity (EPA, 2010). The 2008 EPA assessment also trusted in part on 2003–2004 biomonitoring data available from the National Health and Nutrition Examination Survey (NHANES) which reported measurements of urinary concentrations of triclosan in the U.S. population. Therefore, the 2008 EPA assessment was complete of all triclosan-related exposures (i.e., EPA and FDA regulated uses). The 2008 RED also included new research data on the thyroid effects of triclosan in laboratory animals made available through the EPA‘s Office of Research and Development (ORD). The ORD studies on the thyroid and estrogen results led the EPA to regulate that additional research on the potential health consequences of endocrine effects of triclosan is necessary. This

research is underway and will help characterize the human relevance and potential risk of the results observed from initial laboratory animal studies. The EPA acted to prevent the manufacturer of Playskool toys, Hasbro, Inc. (which sells toys made with Microban® plastic containing triclosan), from making false claims about protecting children from microbial infections in 1997. Hasbro could no longer claim that toys treated with triclosan protect children from infectious diseases caused by bacteria because it did not prove efficacy to EPA. Labels and advertisements for the toys suggested that the treatment protects children from health risks, when in fact it protects only the plastic in the toy. The company is prevented from making such claims due to a lack of reliable data to support them. Under the agreement, Hasbro had to publish large advertisements in certain newspapers and magazines about misrepresentation of the public health claim. On September 2, 2016, after 44 years of its initial proposed rule, FDA delivered a final rule establishing that 19 active ingredients, including triclosan and triclocarban, used in over-the-counter (OTC) consumer antiseptic products intended for use with water (aka consumer antiseptic washes) are not generally recognized as safe and effective (GRAS/GRAE) and are misbranded, and are new drugs for which approved applications under section 505 of the FD&C Act are required for marketing (Safety and Effectiveness of Consumer Antiseptics, 2016).

1.12 Objectives:

The worldwide occurrence of multi drug resistant bacterial strains is a noteworthy concern today. Liquid hand-wash is an important sanitizer to clean our hands and helps us to protect from infection & pathogen. But few ingredients of the hand-wash are really threatening for us and these ingredients can give rise to a group of bacteria that can be resistant to the antimicrobial agents. Therefore, it is significant to find out the effects of liquid hand-wash having triclosan on bacteria and its relation with antibiotic resistance.Although most evidence supports the notion that triclosan increases resistance to antibiotics, this is not necessarily true for all classes of antibiotics. So, the purpose of our study is to

- find out the effects of liquid hand-wash having triclosan on selected clinical isolates and
- its relation with antibiotic resistance.

Chapter 2: Materials & Methods

2.0 Materials and Methods:

The study was carried out in the laboratory of the Department of Mathematics and Natural Sciences at BRAC University.

2.01 Sample collection:

The liquid hand-wash sample of renowned manufacturer used for the study was purchased from standard cosmetics and pharmacy stores in Dhaka city. The batch numbers, expiry dates and the presence or absences of the manufacturers seal were also noticed and recorded.

2.02 Preparation of stock solution of liquid hand-wash:

The liquid hand wash soap sample contain Sodium laureth sulfate (*SLES*),cocamidopropyl betaine,coco diethanolamide, triclosan, benzophenone 3, sorbitol, citric acid, sodium chloride, perfume, preservative, CI 42090, CI 60730, aqua. Firstly, 50ml liquid hand-wash soap was poured in a beaker and then added 50ml distilled water. So, it can be said that a stock solution of 2^{-1} was prepared.

2.03 Microbial cultures used in the study:

Eight clinical isolates were taken from the laboratory. They were *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Salmonella typhi, Proteus vulgaris, Shigella flexneri, Bacillus subtilis, Staphylococcus aureus.*

2.04 Determination of MIC:

In broth micro-dilution method (Lee and Mary, 2013) a mixture of nutrient broth, stock solution and microorganisms of 10 ml volume was prepared in test-tubes. Here, the broth and stock solution constitute 9 ml and the rest 1 ml was for the microorganisms cultured in nutrient broth. Different concentration of stock solution that are used in MIC determination are described in Table:1.

Table 1: Mixture composition in the test-tubes.

Hand-soap(ml)	Nutrient-Broth(ml)	Sample clinical isolates (ml)
0.2 ml	8.8 ml	1ml
0.4 ml	8.6 ml	
0.6 ml	8.4 ml	
0.8 ml	8.2 ml	
1.0 ml	8.0 ml	
1.2 ml	7.8 ml	
1.4 ml	7.6 ml	
1.6 ml	7.4 ml	
1.8 ml	7.2 ml	
2.0 ml	7.0 ml	
2.2 ml	6.8 ml	
2.4 ml	6.6 ml	
2.6 ml	6.4 ml	
2.8 ml	6.2 ml	
3.0 ml	6.0 ml	
3.2 ml	5.8 ml	
3.4 ml	5.6 ml	
3.6 ml	5.4 ml	
3.8 ml	5.2 ml	
4.0 ml	5.0 ml	

Steps in MIC determination:

1. A test-tube containing nutrient broth with desired microorganism and a test-tube with liquid stock solution were taken and treated as control to see the growth.

2. At first the liquid hand-soap and the broth were pipetted into test-tubesin the laminar air-flow cabinetwith 1 ml of desired microorganisms were pipetted from the cultured broth into the test-tubes. Then all the test-tubes were incubated in 37°C in the incubator for 24

hrs. And after 24hrs the test-tubes were observed with comparing with the control and MIC (Minimum Inhibitory Concentration) value was obtained and noted.

3. All the steps were performed in the Laminar Air Flow Cabinet.

2.05 Determination of MBC:

1.After having the MIC value, all the test-tubes were taken inside the laminar air-flow cabinet and streaking was done in nutrient agar petri-plates.

2. Streaking was done for every hand-soap concentration. After streaking the petri-plates were incubated in 37°C in the incubator for 24 hrs. After 24hrs the streaked plates having media were observed for the growth of the desired microorganisms. And a MBC (Minimum Bactericidal Concentration) value was determined and noted.

2.06 Antibiotic susceptibility testing of clinical isolates:

Kirby-Bauer disc diffusion method was used (Bauer et al., 1966) to determine the susceptibility of clinical isolates. Following commercial antibiotics discs were used in the test:

- Ciprofloxacin (5 μg)
- Gentamicin (10 μg)
- Kanamycin (10 μg)
- Nalidixic acid (30 μg)
- Penicillin (10 μg)
- Streptomycin (10 μg)
- Tetracycline (30 μg)
- Erythromycin (15 μg)
- Chloramphenicol (30μg)

Steps performed in antibiotic susceptibility test:

The steps of the work are given beneath:

1. Standardized inoculum of 0.5 McFarland (approximate cell count density: 1.5x108) turbidity standard was prepared by taking 1-2 colonies of organisms.

2. Using sterile cotton swabs, each of the test bacterial strains were lawn cultured on properly labelled Mueller Hinton Agar plates.

4. After lawn culture, sterile forceps were used to carefully pick up antibiotic disks from the stacks and were placed very carefully on the lawn culture.

5. Care was taken to ensure that the disks are well-spaced in order to prevent overlapping of inhibition zones.

6. Then the plates were incubated at 37°C for 24 hours.

7. After incubation antibiotic susceptibility was determined by measuring the diameter of inhibition zone in millimeter.

8. Finally, result was interpreted according to the table given below (table:2).

Table. 2: Chart used in the result interpretation of antibiotic susceptibility testing.

Antibiotic list	**Range (mm)**		
	S	I	R
Chloramphenicol	≥18	13-17	≤12
Kanamycin	≥18	14-17	≤12
Penicillin	≥29	27	≤26
Nalidixic Acid	≥13	11-12	≤10
Streptomycin	≥15	12-14	≤11
Gentamicin	≥15	12-14	≤12
Tetracycline	≥19	15-18	≤14
Erythromycin	≥23	14-22	≤13
Ciprofloxacin	≥21	16-20	≤15

Note: S = Sensitive, R = Resistant, I = Intermediate, (-) = No zone of inhibition

Chapter 3: Results

3.0 Observation &Results:

3.01 Minimum Inhibitory Concentration (MIC) Test Result:

Minimum Inhibitory Concentration was determined by broth micro dilution method (Lee and Mary, 2013) against eight different organisms and their MIC value was obtained.

Table.3: Minimum Inhibitory Concentration of clinical isolates:

Microorganism	MIC value
Pseudomonas aeruginosa	2.4 mlof stock solution
Klebsiella pneumoniae	2.6 ml of stock solution
Escherichia coli	2.0 ml of stock solution
Salmonella typhi	3.0 ml of stock solution
Proteus vulgaris	1.0 ml of stock solution
Shigellaflexneri	0.8 ml of stock solution
Bacillus subtilis	0.6 ml of stock solution
Staphylococcus aureus	0.6 ml of stock solution

Here, *Salmonella typhi*showed the highest MIC value i.e. in 3.0 ml of stock solution and *Bacillus subtilis, whereas Staphylococcus aureus* had shown the lowest MIC value i.e. in 0.6 ml of stock solution.Besides, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris, Shigellaflexneri*had shown the values 2.6 ml, 2.4 ml, 2.0 ml, 1.0 ml, 0.8 ml respectively. The MIC value was observed by their turbidity in the test-tube which was compared with the turbidity of the control.

Fig.1: MIC test of *Proteus vulgaris using broth micro dilution method. The first and the last test-tube in the figure were control.*

3.02 Minimum Bactericidal Concentration (MBC) Test Result:

After obtaining the broth dilution MIC values, MBC value was obtained by using broth microdilution method (Lee and Mary, 2013). The results of MBC test are mentioned in the table3:

Table. 4: Minimum Bactericidal Concentration Test:

Microorganism	MBC value
Pseudomonas aeruginosa	2.8 ml of stock solution
Klebsiella pneumoniae	3.2 ml of stock solution
Escherichia coli	2.6 ml of stock solution
Salmonella typhi	3.8 ml of stock solution
Proteus vulgaris	3.4 ml of stock solution
Shigella flexneri	3.2 ml of stock solution
Bacillus subtilis	3.0 ml of stock solution
Staphylococcus aureus	3.6 ml of stock solution

Here, the highest MBC value was observed for *Salmonella typhi* (3.8 ml of stock solution) and the lowest MBC value was for *Escherichia coli* (2.6 ml of stock solution). *Shigella Flexneri* and *Klebsiella pneumoniae* showed same MBC value (3.2 ml of stock solution).*Staphylococcus aureus*, *Proteus vulgaris*, *Bacillus subtilis* and *Pseudomonas aeruginosa* had MBC value of 3.8 ml, 3.4 ml, 3.0 ml and 2.8 ml of stock solution respectively.

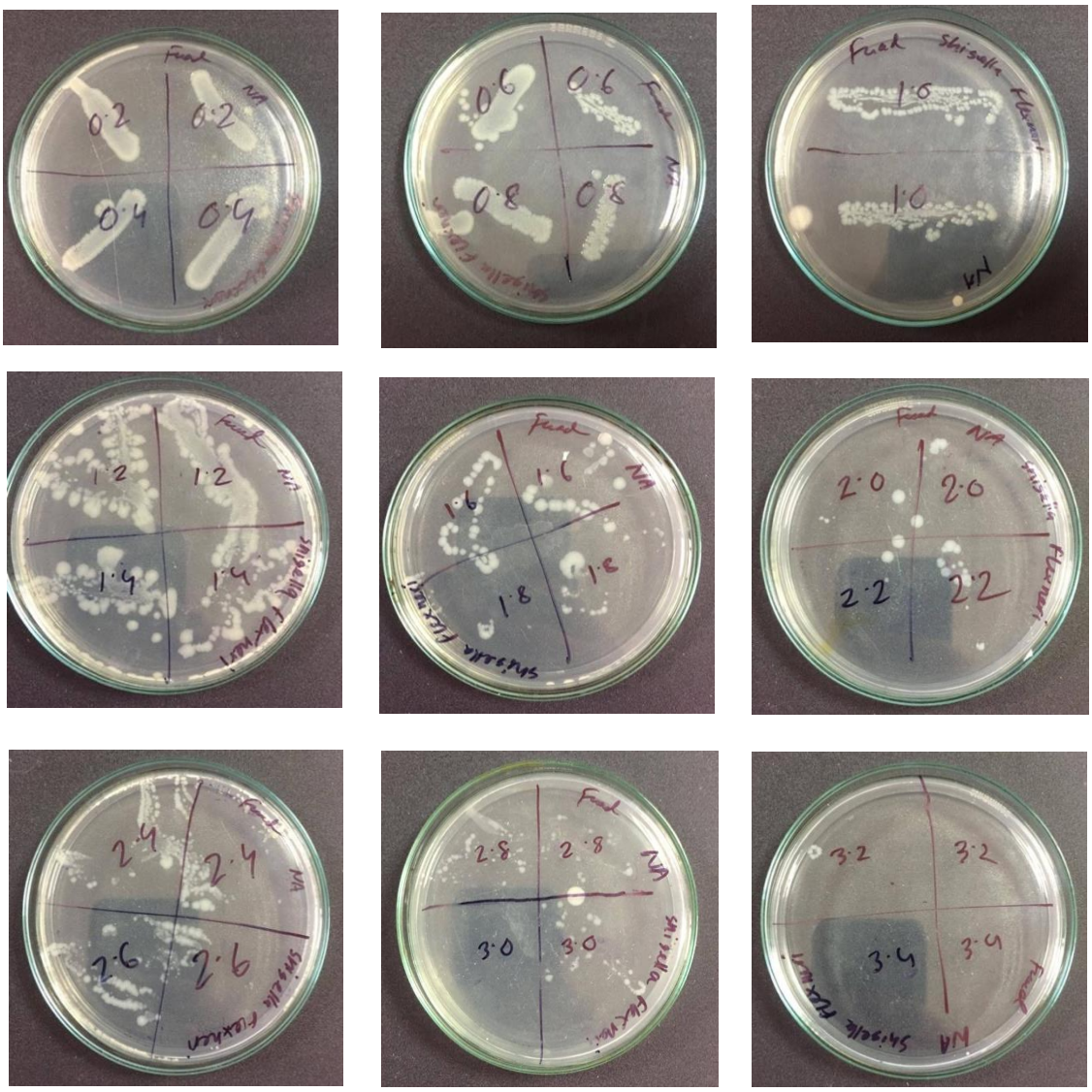

Fig.2: *Shigella flexneri* in nutrient agar media to determine MBC. The figure indicates that the MBC value was 3.2 ml of the stock solution.

Graph of MIC and MBC Value

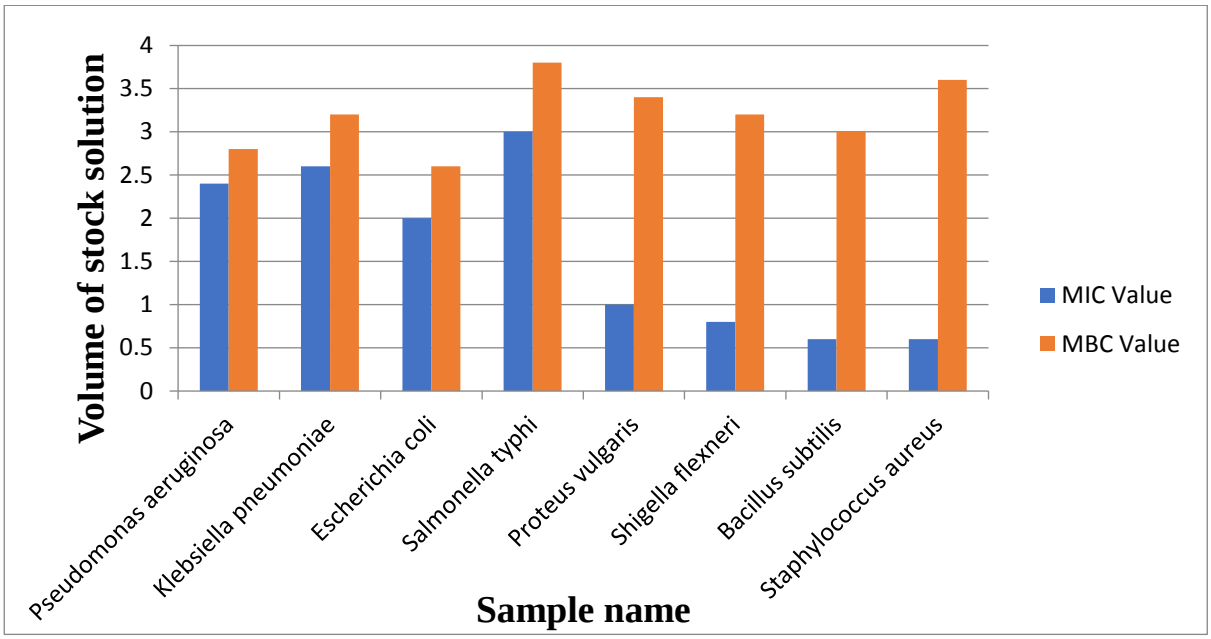

Fig.3: Graph of MIC and MBC value.

3.03 Antibiotic sensitivity test results:

In order to determine the antibiotic sensitivity pattern of the test organisms, nine different antibiotics were used in Kirby-Bauer disc diffusion test (Bauer et al., 1966). It was found that most organisms were resistant to multiple antibiotics. However, few organisms showed susceptibility to antibiotics as well. All of the clinical isolates taken for the study had shown resistant characteristics against at least six tested antibiotics and three isolates were resistant to all the nine antibiotics. It might be possible that some bacterial strain become resistant which leads to their survival even at higher concentration of soaps and antibiotics.

Table.5: Antibiotic Susceptibility Test Result:

Antibiotic list	S-1	In	S-2	In	S-3	In	S-4	In	S-5	In	S-6	In	S-7	In	S-8	In
Chloramphenicol	12	R	12	R	12	R	5	R	12	R	12	R	-	R	14	I
Kanamycin	10	R	10	R	9	R	-	R	10	R	11	R	-	R	10	R
Penicillin	10	R	-	R	16	R	-	R	16	R	17	R	-	R	-	R
Nalidixic Acid	5	R	3	R	-	R	6	R	6	R	-	R	-	R	-	R
Streptomycin	11	R	5	R	12	I	-	R	8	R	10	R	8	R	10	R
Gentamicin	12	I	12	I	15	S	10	R	12	R	12	R	11	R	11	R
Tetracycline	14	R	-	R	5	R	-	R	14	R	10	R	-	R	-	R
Erythromycin	7	R	-	R	23	S	5	R	15	I	-	R	5	R	-	R
Ciprofloxacin	12	R	16	I	15	R	8	R	16	I	13	R	12	R	15	R

Note:

S=Sensitive, R=Resistant, I=Intermediate, -= No zone of inhibition

Sample 01- *Klebsiella pneumoniae*

Sample 02- *Escherichia coli*

Sample 03- *Bacillus subtilis*

Sample 04- *Shigellaflexneri*

Sample 05- *Staphylococcus aureus*

Sample 06- *Salmonella typhi*

Sample 07- *Pseudomonas aeruginosa*

Sample 08- *Proteus vulgaris*

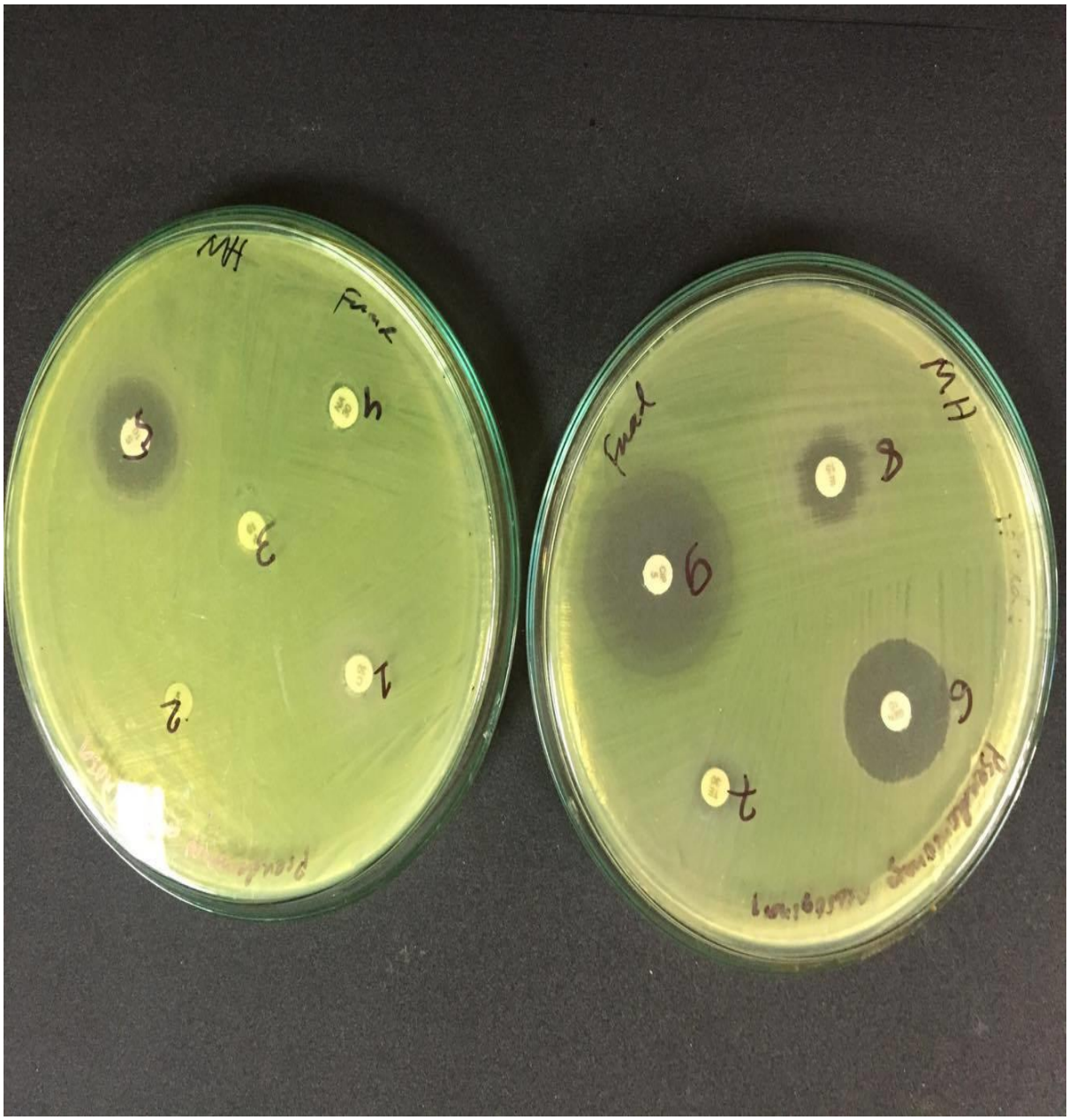

Fig.4: Antibiotic susceptibility test of *Pseudomonas aeruginosa.*

Graph of antibiotic susceptibility test:

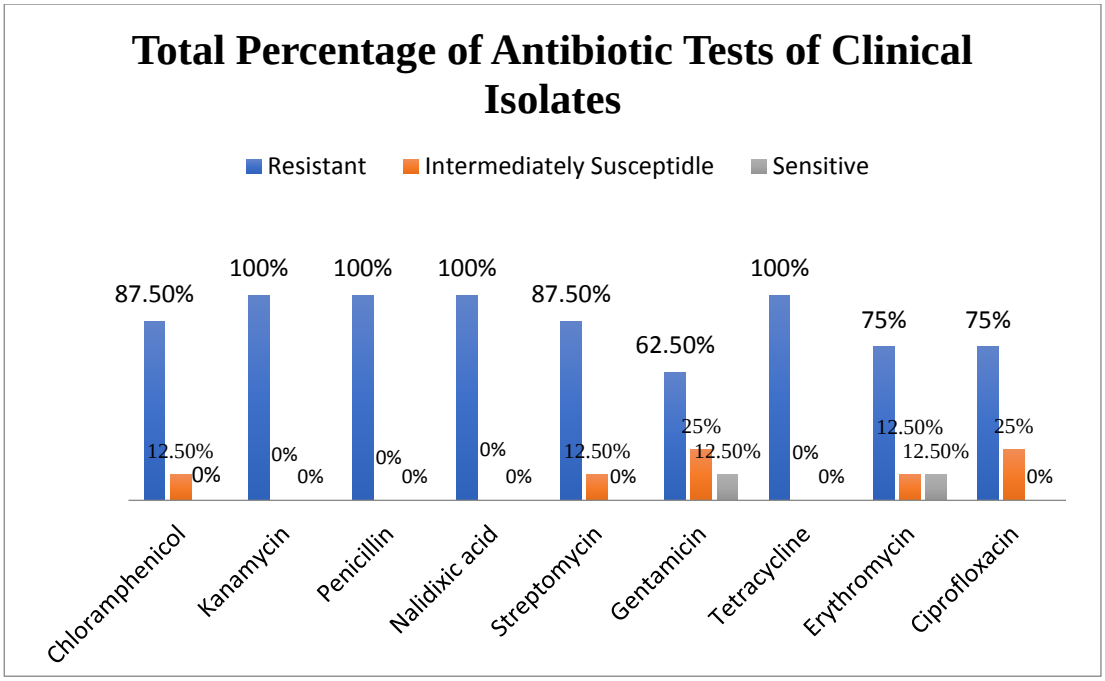

Fig.5: Graph of antibiotic susceptibility test result of clinical samples.

Chapter 4: Discussion

4.0 Discussion:

Washing hands to clean the pathogens (bacteria, fungi, protozoa, helminthes or viruses) and chemicals which can cause personal harm or disease is especially vital for people who handle food or work in the medical field, but it is also an important practice for the general public. For effective hand washing, the application of water alone is inefficient for cleaning skin because water is often unable to remove fats, oils, and proteins, which are components of organic soil. (www.hi-tm.com, 2009). Therefore, removal of microorganisms from skin requires the addition of soaps or detergents to water. Use of liquid soaps have wider acceptability because solid soap due to its reusable nature, may hold bacteria acquired from previous uses, so it is important to wash the soap itself before and after use.(www.pubmedcentral.nih.gov, 2009). However, antibacterial soaps and washes contain common antibacterial agents such as triclosan, chlorhexidine gluconate which has an extensive list of resistant strains of organisms (Westergren and Emilson, 1980) (Tattawasart et al, 1999) (Thomas et al, 2000). So, even if antibacterial soaps and washes aren't selected for antibiotic resistant strains, they might not be as effective as they are marketed to be. Few ingredients of the hand-wash are really threatening for us and these ingredients can give rise to a group of bacteria that can be resistant to the antimicrobial agents. It is important to find out the effects of liquid hand-wash having triclosan on clinical isolates and its relation with antibiotic resistance. Although most evidence supports the notion that triclosan increases resistance to antibiotics, this is not necessarily true for all classes of antibiotics.

The sample of organisms which were used in this investigation were collected and isolated from clinical samples. The liquid hand-wash that we used in our study contains antimicrobial agent triclosan. Triclosan has a large range of bactericidal activity that includes many types of microorganisms but not on all types of microorganisms like gram-negative and gram-positive non-sporulating bacteria, some fungi (Schweizer,2001), *Plasmodium falciparum* and *Toxoplasma gondii* (McLeod et al., 2001). There are many microorganisms that are very sensitive to triclosan like *Staphylococci*, some *Streptococci*, *E. coli*, some mycobacteria and *Proteus* spp. Besides methicillin-resistant *Staphylococcus aureus* (MRSA) strains are also highly sensitive to triclosan (Al-Doori et al.,2003). Patients bathing with 2% triclosan is a potential regimen for decolonization of patients whose skin

is carrying MRSA (Tuffnell et al.,1987). *Pseudomonas aeruginosa* is highly resistant to triclosan and enterococci species like *Klebsiella pneumoniae, Escherichia coli* are usually less susceptible than staphylococci (Russel, 2003).

Bacillus subtilis is a gram-positive bacteria and triclosan is particularly active against gram-positive bacteria (Josephine and Martin, 2002). Mutations of the FabI gene, which lead to alterations of the affinity of triclosan to the active site of FabI and consequent resistance to triclosan, have been identified previously in *Bacillus subtilis* (Heath et al., 2000) (Sivaraman et al., 2003). *Bacillus subtilis* used in the research purpose have MIC value in 0.6 ml and MBC value in 3.0 ml of stock solution. It had the lowest MIC value along with *Staphylococcus aureus* but its MBC value was lower than *Staphylococcus aureus* (3.6 ml of stock solution). In disc-diffusion test, it showed no zone against nalidixic acid. It had shown zones of inhibition against other antibiotics. It was intermediately susceptible against streptomycin and sensitive against gentamicin and erythromycin. In the study, no bacteria had shown sensitivity to any antibiotics except *Bacillus subtilis.*

Isolates of *Staphylococcus aureus*, which had MICs to triclosan between 0.025 and 1 mg/L, were resistant to multiple antibiotics (Suller and Russell, 2000). They showed increased resistance to gentamicin, erythromycin, penicillin, rifampicin, nalidixic acid, tetracycline, methicillin, mupirocin, and streptomycin (Carey and McNamara, 2015). In the research, *Staphylococcus aureus* had given MIC value in 0.6 ml and MBC value in 3.6 ml of the stock solution. It was intermediately susceptible to ciprofloxacin and erythromycin. But the rest shows resistance properties against the antibiotics. *Staphylococcus aureus* strains are also highly sensitive to triclosan (Al-Doori et al.,2003). Triclosan exhibits excellent activity against *Staphylococcus aureus* and issued to control the carriage of meticillin-resistant *S. aureus* (MRSA) in hospitals[shampoo or bath additive with 2% (20g/L) triclosan] (Coia et al., 2006). It has been suggested that hospital treatments may contribute to decreased susceptibility to triclosan. Cookson et al. isolated *Staphylococcus aureus* strains for which triclosan MICs were 2 to 4 μg/ml from patients who had been treated with daily triclosan baths (Cookson et al., 1991).

Infections with *Escherichia coli* usually originate from the person affected (autoinfection), but strains with a particular resistance or disease-causing properties can also be transmitted from animals, through the food chain or between individuals. Levy showed that the potency of triclosan against *Escherichia coli* was reduced by 10- to 20-fold in a soap

formulation (Levy, 2001). Triclosan-adapted *E. coli* exhibited increased resistance to chloramphenicol, tetracycline, amoxicillin, amoxicillin/clavulanic acid, trimethoprim, benzalkonium chloride, and chlorhexidine, while triclosan-adapted *E. coli* O55 exhibited resistance to only trimethoprim (Braoudaki and Hilton, 2004). *Escherichia coli* had MIC value in 2.0 ml and MBC value in 2.6 ml and it was the lowest MBC value found in the test. It did not give any zone of inhibition against penicillin, tetracycline and erythromycin. So, it was highly resistant against these antibiotics. *Escherichia coli* had shown zone of inhibition against other antibiotics which were used. It was intermediately susceptible against gentamicin and ciprofloxacin. *Staphylococcus aureus* was comparatively weaker because it had given zones to all the antibiotics and was intermediately susceptible but *E. coli* did not give any zones against three antibiotics. *Bacillus subtilis* was intermediately susceptible against streptomycin and sensitive against gentamicin and erythromycin. The ability of *E. coli* to acquire genetic resistance to triclosan and related compounds through mutations in the FabI gene indicates that the widespread use of this drug could lead to the appearance of resistant organisms (Cookson et al., 1991).

In my investigation *Proteus vulgaris* had been used and it had given a MIC value in 1.0 ml of the stock solution and MBC value in 3.4 ml of the stock solution. It showed complete resistance against all the tested eight antibiotics. The antibiotic test with chloramphenicol only had shown intermediately susceptible. The MIC value was quite higher than *Staphylococcus aureus* and *Bacillus subtilis* but it had shown more resistant properties against antibiotics.

In this study, *Pseudomonas aeruginosa* had shown MIC value 2.4 ml and MBC value 2.8 ml of the stock solution. It exhibited resistance to all the nine antibiotics. As *Pseudomonas aeruginosa* is highly resistant to triclosan, in the study it has also shown a good MIC and MBC value along with high resistance to antibiotics. *Pseudomonas aeruginosa* is an opportunistic pathogen notable for its high level of resistance to antimicrobial agents (Driscoll et al., 2007). The intrinsic multidrug resistance of *P. aeruginosa* is mediated by a combination of measures including an outer membrane with low permeability and the expression of tripartite multidrug efflux systems belonging to the resistance nodulation division family such as MexAB-OprM and MexXY-OprM (Hancock, 1998) (Poole, 2001). Exposure to triclosan selected for up-regulation of these efflux systems due to mutations in the regulatory gene, *nfxB*, which increased the tolerance to tetracycline, ciprofloxacin,

trimethoprim, erythromycin, and gentamicin. In some cases, the triclosan resistant strains could tolerate up to 500-fold higher antibiotic concentrations than the non triclosan resistant strains (Carey and McNamara, 2015).

The MICs for *Klebsiella pneumoniae* isolates gathered from industrial sources (where triclosan exposure was likely) ranged from 0.1 to 1 μg/ml (Lear et al., 2002). In contrast, the triclosan MICs for 38 and 33% of the *K. pneumoniae* isolates in study were ≥2 and ≥64 μg/ml, respectively (Cole et al., 2003). In this investigation, it is found that *Klebsiella pneumoniae* had shown a MIC value of 2.6 ml of the stock solution. In disc diffusion tests, it was only intermediately susceptible against gentamicin and fully resistant against the other eight antibiotics. In our investigation *Shigella flexneri* had given MIC value of 0.8 ml and had shown resistance against all the antibiotic. *Shigella flexneri* and *Klebsiella pneumoniae* showed same MBC value (3.2 ml of stock solution). In developing countries, the predominant species is *S. flexneri*, which is characterized by long-term persistence of sub-lineages in shigellosis-endemic regions with inadequate hygienic conditions and unsafe water supplies (Connor et al., 2015). Besides ciprofloxacin, the third-generation cephalosporin ceftriaxone is recommended as an alternative for the treatment of shigellosis (WHO, 2005).

Salmonella are associated with much higher mortality than antibiotic-susceptible strains, with quinolone resistance a particular risk factor (Helms et al., 2002).Recent work with *Salmonella* has shown that development as a biofilm provides increased protection against the action of triclosan (Tabak et al., 2007). The MIC and MBC value of *Salmonella typhi* are 3 ml and 3.8 ml of the stock solution and it had the highest MIC and MBC value and showed resistant characteristics against all the tested antibiotics. So, it can be said that the *Salmonella typhi* used in the investigation was highly resistant to hand-soap and antibiotics.

Therefore, all the bacteria used for the research were resistant to most of the antibiotics and higher MIC and MBC value. Actually, all the bacteria used in the study were collected from clinical samples and it may be the main reason for showing high resistance to antibiotics. It is of great importance to know the concentrations of triclosan that select for resistance. It is also necessary to know the communities that are most vulnerable to resistance caused by triclosan. Besides it is equally important to know if resistance caused by triclosan is reversible. Mitigated use of triclosan has been proposed in the U.S. in part

because of the potential concerns over antibiotic resistance (Landau and Young, 2014). Experiments should be performed where triclosan is slowly increased to encourage triclosan-resistance and the mesocosms should be operated at steady-state with a constant supply of triclosan. The resistance profile can then be quantified after triclosan is washed out to determine if triclosan-derived resistance will decrease as triclosan levels decrease. This set of experiments would help to determine the potential impacts of reducing triclosan from environmental system.

The results of this investigation contribute a credence to the use of liquid soap containing triclosan in treating microbial contamination and show that triclosan could be a probable reason for growing antibiotic resistance. Use of alcohols in soap can be an alternative to triclosan. It can be hoped that studies like this will contribute to the establishment of triclosan free liquid hand-wash-soap that could be used for our hygiene purposes. This finding implies that removing triclosan from environmental systems through improved treatment processes or reduced consumer usage could lead to a decrease in triclosan resistance. Regular detection and epidemiological monitoring of both low- and high-risk populations is important. There are growing worries about the emergence and spread of triclosan residues in the environment and its potential negative effect on human and animal health. However, the scientific debate continues regarding the safety and effectiveness of its application in personal care and household products. Triclosan has several important medical uses, and the future aim must be to retain these applications while eliminating the more frivolous and unnecessary ones. It would be wise to restrict the use of triclosan to areas where it has been shown to be effective and most needed.

Research gap and future endeavor:

However, further research is needed for process standardization and optimization. Research is also needed to identify, isolate the chemicals that can have similar properties like triclosan and affect us. In future, we need to study on the following issues:

I. The main limitation of our study was that we included only a single brand of liquid hand wash which is the most commonly available.
II. We did not compare the result with the non-antibacterial handwashes.
III. Here, the investigation was done with eight different pathogens. So, more work should be done with more different pathogens.
IV. Concentration of triclosan required to select for resistance in various environmental communities is still unknown.

Conclusion:

In the rays of current problems with multiple antibioticresistance in clinical strains and the probability for rising resistance to biocides (e.g. triclosan) whose use is growing in the community and it is clear that sensible use ofavailable and as yet effective antimicrobials is called for. Nevertheless, resistance is a common theme in infectionand one that is unlikely to disappear soon. For this reason,the targeting of resistance mechanisms themselves isacquiring credibility (Wright 2000), with moregeneralized resistance mechanisms such as membraneimpermeability and multidrug efflux possibly favored targets. Due to the observed medicated liquid hand wash effect, it is suggested that irrational and long-time application of these products should be discouraged. It is important that during development of tropical antimicrobial products, a multidimensional approach be adopted. This will ensure that resultant products are designed for specific media of the market and that those needs are met. Ultimately, the product is more likely to have a long, useful, and profitable utilization.

References

1. "FDA Taking Closer Look at 'Antibacterial' Soap". U.S. Food and Drug Administration. (2016)
2. Adolfsson-Erici, Patterson M., Parkkonen J., and Sturve J. (2000). Triclosan, A Commonly Used Bactericide Found in Human Milk and in the Aquatic Environment, in Abstracts of Dioxin, 2000, 20th International Symposium on Halogenated Environmental Organic Pollutants and POP's: Monterey, CA. , Volume 48.
3. Aiello AE, Larson EL, Levy SB. Consumer antibacterial soaps: effective or just risky? Clin Infect Dis 2007;45 Suppl 2: S137–47.
4. Aiello, A., 2008. Consumer antibacterial soaps: effective or just risky? Clin Infect Dis., 1:454 Suppl 2: S137-147.
5. Al-Doori, Z., Morrison, D., Edwards, G. et al. (2003). Susceptibility of MRSA to triclosan. Journal of Antimicrobial Chemotherapy 51, 185–6.
6. *ANTIMICROBIAL RESISTANCE Global Report on Surveillance* (pp. 1-232, Rep.). (2014). Geneva, Switzerland: World Health Organization. doi: http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf
7. B.A. Wilson, V.H Smith, F. de Noyelles Jr. C.K. Larive, Effects of three pharmaceutical and personal care products on natural freshwater algal assemblages, Environ.Sci. Technol. 2003.
8. Baldry P. The battle against bacteria -a fresh look. Cambridge University Press; 1976. p. 156.
9. Bamber, A. I. & Neal, T. J. (1999). An assessment of triclosan susceptibility in methicillin-resistant and methicillin-sensitive Staphylococcus aureus. Journal of Hospital Infection 41, 107–9.
10. Bardhan P, Faruque AS, Naheed A, Sack DA. Decrease in shigellosis-related deaths without Shigella spp.-specific interventions, Asia. Emerg Infect Dis. 2010;16(11):1718–1723. doi: 10.3201/eid1611.090934.

11. *Bauer, AW; Kirby, WM; Sherris, JC; Turck, M (April 1966). "Antibiotic susceptibility testing by a standardized single disk method.". American journal of clinical pathology.* ***45*** *(4): 493–6.* PMID 5325707
12. Braoudaki M., Hilton A. C. (2004). Low level of cross-resistance between triclosan and antibiotics in *Escherichia coli* K-12 and *E. coli* O55 compared to *E. coli* O157. FEMS microbiology letters. 235, 305–309. 10.1016/j.femsle.2004.04.049.
13. Calafat, A.M., Ye, X., Wong, L.Y., Reidy, J.A., Needham , L.L. Urinary concentrations of triclosan in the U.S. population: 2003-2004. Environ Health Perspect. 2008 Mar;116(3):303-7.
14. Carey E. D. and McNamara J.P. (2015). The impact of triclosan on the spread of antibiotic resistance in the environment. NCBI. 5:780. doi: 10.3389/fmicb.2014.00780
15. Coia JE, Duckworth GJ, Edwards DI, Farrington M, Fry C, Humphreys H, et al. Guidelines for the control and prevention of meticillin-resistant Staphylococcus aureus (MRSA) in healthcare facilities. J Hosp Infect 2006;63(Suppl.1):S1–44.
16. Cole, E. C., R. M. Addison, J. R. Rubino, K. E. Leese, P. D. Dulaney, M. S. Newell, J. Wilkins, D. J. Gaber, T. Wineinger, and D. A. Criger. 2003. Investigation of antibiotic and antibacterial agent cross-resistance in target bacteria from homes of antibacterial product users and nonusers. J. Appl. Microbiol.95**:**664-676.
17. Connor TR, Barker CR, Baker KS, Weill F-X, Talukder KA, Smith AM, et al. Species-wide whole genome sequencing reveals historical global spread and recent local persistence in *Shigella flexneri*. Elife. 2015;4:e07335. http://dx.doi.org/10.7554/eLife.07335
18. Cookson, B. D., H. Farrelly, P. Stapleton, R. P. Garvey, and M. R. **Price.** 1991. Transferable resistance to triclosan in MRSA. Lancet337**:**1548-1549.
19. Courtney, K.D.., Moore, J.A., Toxicology and Applied Pharmacology, Vol. 20, 396.
20. Crinnion, W.J. The CDC fourth national report on human exposure to environmental chemicals: what it tells us about our toxic burden and how it assist environmental medicine physicians. Altern Med Rev. 2010 Jul;15(2):101-9. Review.
21. Crump J, Mintz E. Global trends in typhoid and paratyphoid Fever. Clin Infect Dis. 2010;50:241–246.
22. Driscoll, J. A., S. L. Brody, and M. H. Kollef. 2007. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. Drugs67**:**351-368.

23. EPA. Triclosan Facts. Washington, DC: U.S. Environmental Protection Agency; 2010. [accessed 27 September 2017]. Available: http://www.epa.gov/oppsrrd1/REDs/factsheets/triclosan_fs.htm.
24. Fan, F., Yan, K., Wallis, G. S. et al. (2002). Defining and combating the mechanisms of triclosan resistance in clinical isolates of Staphylococcus aureus. Antimicrobial Agents and Chemotherapy 46, 3343–7.
25. FDA. (2016, September 2). FDA issues final rule on safety and effectiveness of antibacterial soaps. Retrieved September 26, 2017, from https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm517478.htm
26. FDA. Triclosan: What Consumers Should Know. Washington, DC: U.S. Food and Drug Administration; 2010. [accessed 27 September 2017]. Available:http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm205999.htm
27. Fiss E.M., Rule K.L., Vikesland P.J. Formation of chloroform and other chlorinated byproducts by chlorination of triclosan-containing antibacterial products. Environ Sci Technol. 2007 Apr 1;41(7):2387-94.
28. Friedman, M and Wolf, R (1996). Chemistry of soaps and detergents various types of commercial products and their ingredient. *Clinical dermatology* 14: 7-13.
29. Glasser, A. Triclosan, the ubiquitous antibacterial agent. Pesticides and You. Beyond Pesticides/National Coalition Against the Misuse of Pesticides. Vol.24, No.3, 2004,12.
30. Gu B, Cao Y, Pan S, Zhuang L, Yu R, Peng Z, Qian H, Wei Y, Zhao L, Liu G, et al. Comparison of the prevalence and changing resistance to nalidixic acid and ciprofloxacin of Shigella between Europe-America and Asia-Africa from 1998 to 2009. Int J Antimicrob Agents. 2012;40(1):9–17. doi: 10.1016/j.ijantimicag.2012.02.005.
31. H. Singer, S. Muller, C. Tixier and L. Pillonel. Triclosan: occurrence and fate of a widely used biocide in the aquatic environment: field measurements in wastewater treatment plants, surface waters, and lake sediments.Environ. Sci. Technol. 2002, 36, 4998-5004.
32. Hancock, R. E. 1998. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. Clin. Infect. Dis.27**:**S93-S99.
33. Hawkey PM. The origins and molecular basis of antibiotic resistance. Brit Med J. 1998; 317:657–660.

34. Heath RJ, Su N, Murphy CK et al. The enoyl-[acyl-carrier-protein] reductases FabI and FabL from Bacillus subtilis. J Biol Chem 2000; 275: 40128–33.
35. Heath, R. J., White, S. W. & Rock, C. O. (2001). Lipid biosynthesis as a target for antibacterial agents. Progress in Lipid Research 40, 467–97.
36. Helms M, Vastrup P, Gerner-Smidt P et al. Excess mortality associated with antimicrobial drug resistant Salmonella Typhimurium. Emerg Infect Dis 2002; 8 : 490–5.
37. Hong HA, Khaneja R, Tam NM, Cazzato A, Tan S, Urdaci M, Brisson A, Gasbarrini A, Barnes I, Cutting SM (March 2009). "Bacillus subtilis isolated from the human gastrointestinal tract". Research in Microbiology. **160** (2): 134–43. PMID 19068230. doi:10.1016/j.resmic.2008.11.002.
38. James MO, Li W, Summerlot DP, Rowland-Faux L, Wood CE. Triclosan is a potent inhibitor of estradiol and estrone sulfonation in sheep placenta. Environ Int. 2010 Nov;36(8):942-9. Epub 2009 Mar 18.
39. Johnson, S.A., Goddard, P.A., Ilife,C.. Timmens, B., Richard., A.H., Robson, G and Handley, P.S (2002). Comparative susceptibility of resident and transient hand bacteria to para-chlorometa-xylenol and triclosan. *Journal of Applied Microbiology*. 93: 336-344
40. Jones, G. L., A. D. Russell, Z. Caliskan, and D. J. Stickler.2005. *A strategy for the control of catheter blockage by crystalline Proteus mirabilis biofilm using the antibacterial agent triclosan.* Eur. Urol.48**:**838-*845.*
41. Josephine, J, B., and Martin C. J Wale. (2002) .The antibacterial activity of triclosan-impregnated storage boxes against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus and Shewanella putrefaciens in conditions simulating domestic use. *Journal of Antimicrobial Chemotherapy,49*(1). Retrieved from https://doi.org/10.1093/jac/49.1.87.
42. Kimel, L.S (1996). Hand washing education can decrease illness absenteeism. *Journal of School Nursery*. 12: 14-18.
43. Kolpin, Dana et al. (2002) Pharmaceuticals, hormones and other organic wastewater contaminants in U.S. Streams, 1999-2000: A National Reconnaissance, Environmental Science and Technology v. 36: 1202-1211.

44. Landau E., Young S. (2014). Minnesota issues ban on antibacterial ingredient. *CNN (online)* Available at: http://www.cnn.com/2014/05/21/health/triclosan-ban-antibacterial/
45. Larson, E,. McGinley, K., Grove, G.L., Leyden. J.J and Talbot, G.H (1989). Physiologic, microbiologic and seasonal effects of hand washing on the skin of health care personnel. *American Journal of Infection control* 14: 5-90.
46. Lear, J. C., J. Y. Maillard, P. W. Dettmar, P. A. Goddard, and A. D. Russell**.** 2002. Chloroxylenol- and triclosan-tolerant bacteria from industrial sources. J. Ind. Microbiol. Biotechnol.29**:**238-242.
47. Lee, Mary (2013). Basic Skills in Interpreting Laboratory Data (5 ed.). ASHP. p. 723.
48. Levy, C. W., Roujeinikova, A., Sedelnikova, S. et al. (1999). Molecular basis of triclosan activity. Nature 398, 383–4.
49. Levy, S. B. 2001. Antibacterial household products: cause for concern. Emerg. Infect. Dis.7**:**512-515.
50. Livermore DM. Lactamases in laboratory and clinical resistance. Clin Microbiol Rev. 1995; 8:557–584.
51. Lyczak, J. B., Cannon, C. L., & Pier, G. B. (2000). Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist. *Microbes and Infection*, *2*(9), 1051–1060. https://doi.org/10.1016/S1286-4579(00)01259-4
52. McDonnell, G., Klein, D., Haines, K., Pretzer, D. The importance of neutralization in the evaluation of triclosan-containing products. Journal of Industrail Microbiology & Biotechnology (1998) 21, 184-186.
53. McLeod, R., Muench, S.P., Rafferty, J.B. et al. (2001). Triclosan inhibits the growth of Plasmodium falciparum and Toxoplasma gondii by inhibition of apicompexan FabI. International Journal of Parasitology 31, 109–13.
54. McMurry LM, Oethinger M, Levy SB. Nature. Triclosan targets lipid synthesis. 1998 Aug 6;394(6693):531-2.
55. Menoutis, J., Parisi-Menoutis, A.I. Technology Review Series, Triclosan and Its Impurities; Quantex Laboratories.
56. Miller, T.L., Lorusso, D.J., Walsh, M.L., Deinzer, M.L. The acute toxicity of penta-, hexa-, and heptachlorohydroxydiphenyl ethers in mice. J Toxicol Environ Health. 1983 Aug-Sep;12(2-3):245-53.

57. Mirza S, Beeching N, Hart C. Multi-drug resistant typhoid: a global problem. J Med Microbiol. 1996;44:317–319.
58. Musser JM. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin Microbiol Rev. 1995; 8:496–514.
59. Nikaido, H. (2010). Multidrug Resistance in Bacteria. *Annu Rev Biochem.*, (2), 119–146. https://doi.org/10.1146/annurev.biochem.78.082907.145923.
60. O'Hara CM *et al.* (2000) Classification, identification, and clinical significance of Proteus, Providencia, and Morganella *Clin Microbiol Rev* 13:534-46.
61. Osborne, R.C and Grube, J (1982). Hand disinfection in dental practice, *Journal of Clinical Preview*. Dent 4:11-15.
62. Pang T, Levine MM, Ivanoff B, Wain J, Finlay BB. Typhoid fever--important issues still remain. Trends Microbiol. 1998;6:131–133.
63. Parry C, Hien TT, Dougan G, White N, Farrar J. Typhoid fever. N Engl J Med. 2002;347:1770–1782.
64. Podschun, R., & Ullmann, U. (1998). Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical Microbiology Reviews*, *11*(4), 589–603. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9767057
65. Poole, K (2002). Mechanisms of bacterial bioade and antibiotic resistance. *Journal of Applied Microbiology*. 92: 555-564.
66. Poole, K. 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J. Mol. Microbiol. Biotechnol.3**:**255-264.
67. Richards, M.J, Edwards,J.E., Culver, D.H and Gaynes, R.P (1999). Nosocomial infections in medical intensive care units in the United States. National Nosocomial infections surveillance system. *Critical Care Medicine journal* 27. 887-892.
68. Roberts JA, Cumberland P, Sockett PN et al. The study of infec-tious intestinal disease in England: socio-economic impact. Epidemiol Infect 2003; 130: 1–11.
69. Robertshaw, H., Leppard, B. Contact dermatitis to triclosan in toothpaste. Contact Dermatitis. 2007 Dec;57(6):383-4.
70. Russell, A. D. (2003). Similarities and differences in the responses of microorganisms to biocides. Journal of Antimicrobial Chemotherapy 52, 750–63.

71. Russell, A. D. (2003). Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. Lancet Infectious Diseases 3, 794–803.
72. Russell, A. D. (2004). Bacterial adaptation and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon. Journal of Hospital Infection.
73. Sabbagh S, Forest C, Lepage C, Leclerc J, Daigle F. So similar, yet so different: uncovering distinctive features in the genomes of Salmonella enterica serovars Typhimurium and Typhi. FEMS Microbiol Lett. 2010 Jan 20; [Epub ahead of print].
74. Safety and Effectiveness of Consumer Antiseptics; Topical Antimicrobial Drug Products for Over-the-Counter Human Use. Federal Register, vol. 81, no. 172. September 6, 2016. https://www.gpo.gov/fdsys/pkg/FR-2016-09-06/pdf/2016-21337.pdf
75. Samsøe-Petersen, L., M. Winther-Nielsen, and T. Madsen, Danish EPA. Fate and Effects of Triclosan. September 2003.
76. Schena, D., Papagrigoraki , A., Girolomoni, G. Sensitizing potential of triclosan and triclosan-based skin care products in patients with chronic eczema. Dermatol Ther. 2008 Oct;21 Suppl 2:S35-8
77. Sivaraman S, Zwahlen J, Bell AF et al. Structure–activity studies of the inhibition of FabI, the enoyl reductase from Escherichia coli, by triclosan: kinetic analysis of mutant FabIs. Biochemistry 2003;42: 4406–13.
78. Slater-Radosti, C., Van Aller, G., Greenwood, R., Nicholas, R., Keller, P., DeWolf, W. E., Jr, Fan, F., Payne, D. J. & Jaworski, D. D. (2001). Biochemical and genetic characterization of the action of triclosan on Staphylococcus aureus .J Antimicrob Chemother 48, 1–6.
79. Suller M. T. E., Russell A. D. (2000). Triclosan and antibiotic resistance in *Staphylococcus aureus*. J Antimicrob Chemother. 46, 11–18. 10.1093/jac/46.1.1.
80. Tabak M, Scher K, Hartog E *et a*l. Effect of triclosan on Salmonella typhimurium at different growth stages and in biofilms. FEMS Microbiol Lett 2007; 267: 200–6.
81. Tattawasart, U., Maillard, J.Y., Furr, J.R. and Russell, A.D. 19999. Development of resistance to chlorhexidine diacetate and cetylpyridinium chloride in Pseudomonas stutzeri and changes in antibiotic susceptibility. Journal of hospital Infection 42:219-229.

82. Tenover, F. C. (2006). Mechanisms of Antimicrobial Resistance in Bacteria. *The American Journal of Medicine*, *119*(6), S3–S10. https://doi.org/10.1016/j.amjmed.2006.03.011.
83. Thomas, L., Maillard, J.Y., Lambert, R.J. and Russell, A.D. 20000. Development of resistance to chlorhexidine diacetate in Pseudomonas aeruginosa and the effect of a "residual" concentration. Journal of Hospital Infection 46:297-303.
84. Tomasz A, Munaz R. b-lactam antibiotic resistance in Gram positive bacteria pathogens of upper respiratory tract: a brief overview of mechanisms. Microbial Drug Resistance. 1995;1: 103–109.
85. Tortora, G. J., Funke, B. R. & Case, C. L. (2010). *Microbiology: An introduction*. Boston: Benjamin Cummings.
86. Triclosan. (2011). *White Paper prepared by The Alliance for the Prudent Use of Antibiotics (APUA).*
87. Tuffnell, D.J., Croton, R.S., Hemingway, D.M., Hartley M.N., Wake P.N., Garvey R.J. (1987). Methicillin resistant Staphylococcus aureus; the role of antisepsis in the control of an outbreak. J Hosp Infect. Nov;10(3):255-9.
88. Vachon M. A report presented by George Soros Open Society Institute. New York: 1999. Harvard Medical School Report Warns of World Health Threat.
89. Van den Berg M, Birnbaum LS, Denison M, et al. The 2005 World Health Organization reevaluation of human and Mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol Sci. 2006 Oct;93(2):223-41. Epub 2006 Jul 7.
90. Van der Straaten T, Janssen R, Mevius DJ et al. Salmonella gene rma (ramA) and multiple-drug-resistant Salmonella enterica serovar Typhimurium. Antimicrob Agents Chemother 2004;48: 2292–4.
91. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, Nouwen JL. 2005. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis5:751–762. doi:10.1016/S1473-3099(05)70295-4.
92. Westergren, G. and Emilson, C.G. 1980. In vitro development of chlorhexidine resistance in Streptococcus sanguis and its transmissibility by genetic transformation. Scandinavian Journal of Dental Research 88:236-243.
93. White, D.G and McDermolt, P.F ((2001). Biocides, drug resistance and microbial evolution; *Current Opinion in Microbiology*; 4: 313-317.

94. Wright, G.D. (2000) Resisting resistance: new chemical strategies for battling superbugs. Chemistry and Biology 7, R127–R132.
95. www.hi-tm.com English SOP. 2009. Standard Operating Procedure. Retrieved on 2009-04-27. www.learnwell.org. 2009. Hand Hygiene for Healthcare Workers. LearnWell Resources, Inc, a California nonprofit public benefit corporation. Retrieved on 2017-10-07.
96. www.pubmedcentral.nih.gov, 2009.

Appendix - I

Media composition

Composition of the media used in this study is provided below. Media was autoclaved at 121°C for 15 min at 121psi.

1.Nutrient Agar (HiMedia, India)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.5
Sodium Chloride	5.0
Yeast extract	1.5
Agar	15.0

2.Mueller-Hinton Agar (HiMedia, India)

Ingredients	Amount (g/L)
Beef infusion	300
Casamino acids	17.5
Starch	1.5
Agar	17.0

3.Nutrient broth

Ingredients	Amount (g/L)
'Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
pH 7.4 (+,-) 0.2@ 25°C	

Appendix-II

Instruments

Autoclave	**Wisd Laboratory Instruments** **Made in Korea**
Incubator	Model: DSI 3000 Digisystem Laboratory Instrument Inc. Made in Taiwan.
Vortex Mixer	Model: VM-2000 Digisystem Laboratory Instrument Inc. Made in Taiwan
Electronic Balance	RADWAG Wagi ELEktroniczne Model: WTB 200
Refrigerator (4°C)	Model: 0636 Samsung