ASSESSMENT OF ANTIMICROBIAL AND COAGULANT ACTIVITIES OF CBRN DECONTAMINATION MATERIALS

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ABSTRACT

ASSESSMENT OF ANTIMICROBIAL AND COAGULANT ACTIVITIES OF CBRN DECONTAMINATION MATERIALS

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CBRN Defense is an emerging interdisciplinary branch of science that combines physics, chemistry and biology together in a unique and complementary way. Concept of decontamination, which is a necessity to neutralize or reduce the spread of contaminants, is one of the key topics in this area of study, and many of the commercially available products are in liquid form and mainly intended to be used for decontamination of environment and equipments. Material used in this study is in powder form that is not dissolvable in liquids, and thus providing a novel procedure for testing antimicrobial effect of such materials as well. Decon powder is intended to be used on human skin for decontamination against CBRN agents, and its effect on blood coagulation for superficial bleedings was also investigated. We applied varying doses of decon powder on *E.coli*, *B.subtilis* and *B.subtilis* endospores and also along with another known antibacterial compound, protamine, to determine the minimum concentration of powder needed to prevent the visible growth of colonies on agar plates, and found that Gram positive bacteria are more easily affected by the decon powder formulation compared to Gram negative

bacteria. Minimum Inhibitory concentration for *B.subtilis* was determined to be 50 mg decon powder, whereas for *E.coli*, it was 100 mg given 60 minutes, and 500 mg given 15 minutes. Decon powder formulation could prevent the activation and significantly reduce the visible growth of endospores with 500 mg dose and 100% reduction in growth was observed at 200 mg decon powder mixture with 50 mg protamine. Furthermore, using whole blood sample, we determined that the application of decon powder could also help reducing the superficial bleedings in vitro compared to standard gauzes, and this effect was found statistically significant.

Keywords: CBRN, Decontamination, CFU, Antibacterial, Coagulation

ÖZ

KBRN DEKONTAMİNASYON MATERYALLERİNİN ANTİMİKROBİYAL VE KOAGÜLANT AKTİVİTELERİNİN BELİRLENMESİ

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KBRN Savunma, gelişmekte olan disiplinlerarası bir bilim dalı olup bünyesinde fizik, kimya ve biyolojiyi birleştiren yeni bir alandır. Dekontaminasyon, KBRN savunma alanının temel çalışma alanlarından biri olup, piyasada mevcut dekontaminant ürünlerin büyük çoğunluğu sıvı halde ve alan ile ekipman dekontaminasyonu üzerine tasarlanmışlardır. Dekontaminasyon toksik materyallerin nötraliasyonu ve etrafa zarar vermeye devam etmesinin engellenmesi açısından elzemdir. Bu çalışmada kullanılan materyal hiçbir sıvıda çözünme özelliğine sahip olmadığı için antimiktobiyal madde testi için yeni bir prosedürün hazırlanmasını gerektirmiştir. Dekontaminasyon malzemesi insan derisi üzerinde KBRN ajanlarının etkisini sınırlandırmak için üretilmiş olup yüzeysel kanamalarda kullanılmak üzere kan pıhtılaştırıcı etkisi de incelenmiştir. Değişen dozlarda uygulanan dekontaminant malzeme *E.coli, B.subtilis* ve *B.subtilis* sporları üzerinde tek başına ve antibakteriyel özelliği bilinen protamin ile birlikte görünür koloni oluşumunu engelleyen en düşük

dozu tespit etmek için denenmiştir. Gram pozitif bakterilerin, Gram negatif bakterilere oranla dekontaminant materyale karşı daha zayıf kaldığı gözlemlenmiştir. Kullanılan dekontaminant materyalin Minimum İnhibitör Konsantrasyonu *B.subtilis* için 50 mg, *E.coli* için 60 dakikalık kontakt süresinde 100 mg, 15 dakikalık kontakt süresinde ise 500 mg olarak belirlenmiştir. Sporlar üzerinde ise 500 mg doz kullanılarak görünür koloni büyümesi büyük oranda engellenmiş olup, 250 mg dekontaminant ve protamin karışımında ise koloni büyümesinde %100'lük azalma gözlenlenmiştir. Buna ek olarak, tam kan örneği kullanarak dekontaminant materyalin deri üzerinde yüzeysel kanamalarda kullanılmak üzere etkinliği in vitro olarak standart gazlı bezlere göre daha efektif olduğu istatistiksel olarak gösterilmiştir.

Anahtar Kelimeler: KBRN, Dekontaminasyon, Antibakteriyel, CFU, Koagülasyon

To my family…

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And finally, in this galaxy or any other, may the force be with us all…

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ABBREVIATIONS

AgNPs: Silver nanoparticles

BWA: Biological Warfare Agent

CBRN: Chemical, Biological, Radiological and Nuclear

CDC: Centers for Disease Control and Prevention

CFU: Colony Forming Unit

CWA: Chemical Warfare Agent

Decon: Decontamination

HAZMAT: Hazardous Materials

LBA: Luria Bertani Agar

MIC: Minimum Inhibitory Concentration

RWA: Radiological Warfare Agent

SD: Standard deviation

TiO_2: Titanium dioxide

ZnO: Zinc oxide

CHAPTER 1

INTRODUCTION

1.1 CBRN Defence

The threat to all life forms on earth posed by the weapons of mass destruction has never been more apparent and frightening than in the past decades. Emergence of thermonuclear bombs (second generation atomic bombs), which can be thousands of times more powerful than conventional nuclear bombs, has led the probability of a world-wide catastrophe to an alarming degree (Gsponer, 2008). Having said that, modern world's advanced warfare arsenals reach beyond the radioactive threats. Biological and chemical weapons are no less of a threat to the human kind. Specially, airborne bioweapons can cause millions of people to suffer dire consequences while leaving the environmental infrastructure intact, which makes them, in theory, preferable choice of weapons in the eyes of terrorist organizations and authoritarion states. Although CBRN agents are generally hard to detect before the attack occurs, as there is need for intelligence to give autohorities sufficient time to mobilize measures for all contingincies, decontamination of such agents are of cruical importance in order to cope with the aftermath (Frischknecht, 2003). Rapid and effective decontamination not only limits the spread of contaminants, but also reduces the mortality and morbidity. During such incidents, decontaminating victims (affected individuals) should be number one priority.

1.1.1 CBRN Agents

1.1.1.1 Chemical Warfare Agents (CWAs)

Chemical warfare agents are toxic subtances that are designed and weaponized specifically to injure, incapacitate or kill an enemy or civilian targets and associated with military operations and terrorist organizations (Chauhan et al, 2008). Although their use is prohibited by the Chemical Weapons Convention signed in 1993, and until today 193 states signed it, the threat of chemical weapons still remains as there is an undeniable possibility that totalitarian governments and terrorist organizations around the globe can still produce and be in possession of such agents. Chemical warfare agents can be in solid, liquid or gaseous form, and have been classified into blister agents, nerve agents, choking agents, incapitating agents and blood agents by North Atlantic Treaty Organization (NATO) (Table 1.1), although there may be differences in classification in literature.

Table 1.1 Classification of CWAs (Taken from Polat et al, 2018)

Blister agents	Nerve agents	Choking agents	Asphyxiants/ Blood agents	Behavioural agents/ Incapatitating
Sulfur mustard Nitrogen mustard Lewisite	G Series • Sarin • Soman • Tabun V Series	Phosgene Diphosgene Chlorine Chloropicrin	Hydrogen cyanide Cyanogen chloride Arsine	Lysergic acid Diethylamide (LSD-25) Ketamine

Throughout the history, various CWAs have been used in wars and against civilian populations (Figure 1.1). According to their classification, different CWAs have different effects and purposes to be used against targets.

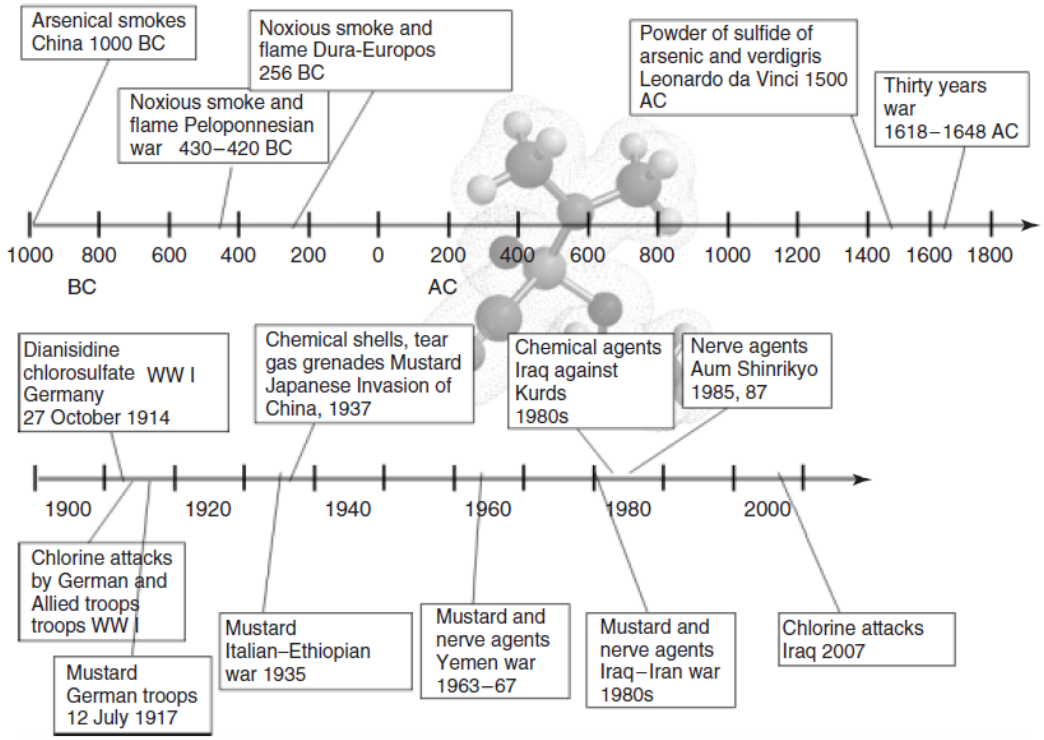

Figure 1.1 Examples of the use of CWAs throughout history (Taken from Richardt et al, 2013)

Nerve agents, which are sarin, soman, tabun and VX, are organic compounds that do not exist naturally, are found in liquid form, and belong to organophosphate group of chemicals. These toxic compounds disrupt the regular functioning of the nervous

system by inhibiting the enzyme acetylcholinesterase which is a cholinergic enzyme that breaks down acetylcholine in nervous system (Figure 1.2). Its inhibition results in excess accumulation of this neurotransmitter in synaptic cleft and causes various symptoms such as bronchoconstriction, miosis, diarrhoea, agitation and involuntary muscle contraction (Geoghegan & Tong, 2006).

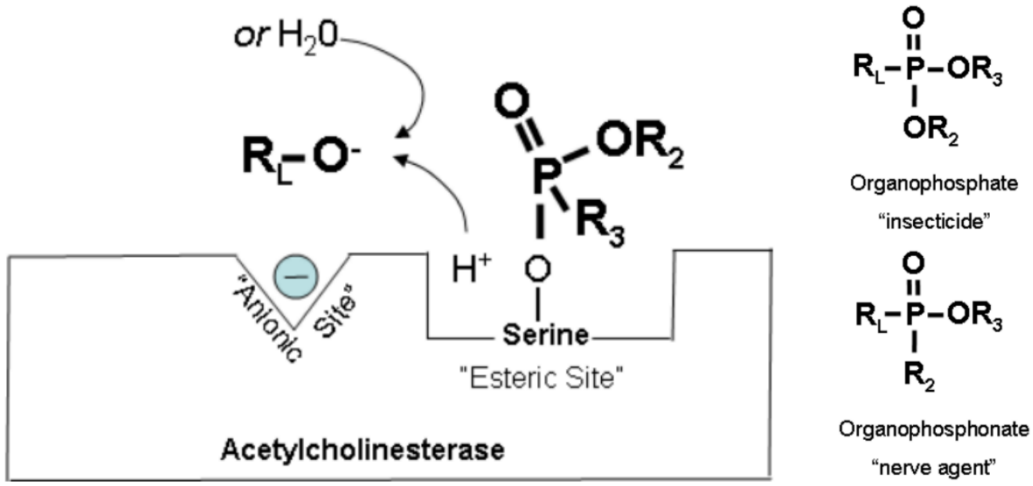

Figure 1.2 Inhibition of Acetylcholinesterase by nerve agent (Taken from https://www.atsdr.cdc.gov/csem/cholinesterase-inhibitors/inhibitors.html)

Blister agents (also known as vesicants) are toxic chemicals that cause blistering on skin when come in contact with. These include lewisite, sulfur mustard, and nitrogen mustard. Although not deadly unless exposed to large quantities, blister agents can be used mainly to incapacitate an enemy and to overload health care services specially in a war, causing prolonged morbidity These alkylating agents can also affect eyes and respiratory system (Polat et al, 2018). There are no known antidotes

for sulfur and nitrogen mustard, however for lewisite, an antidote known as British Anti-Lewisite (2,3-dimercaptopropanol) exists (Figure 1.3).

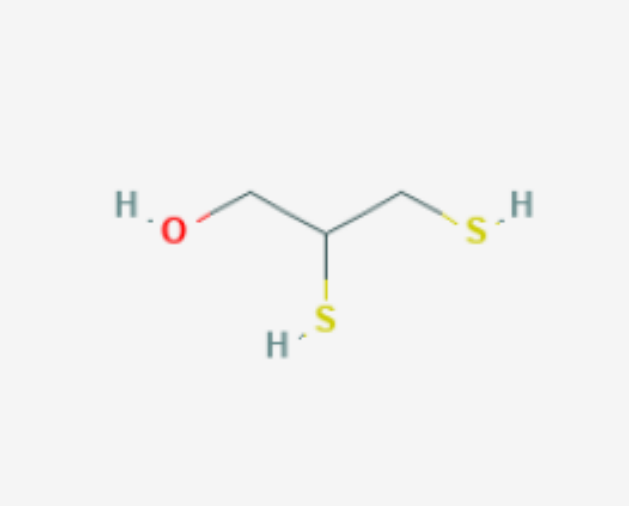

Figure 1.3 British Anti-Lewisite *Lewisite (Taken from https://www.cdc.gov/NIOSH/ershdb/EmergencyResponseCard_29750006.html)*

Choking agents such as phosgene and chlorine exert their effect on the lungs, causing respiratory distress. The effect on the lungs can be permanent depending on the duration and amount of the gas exposed. Typical symptoms include pulmonary edema and reduced pulmonary compliance (Shea, 2013). There are no antidotes for choking agents, main cause of death from exposure is respiratory failure.

Blood agents or asphyxants are metabolic poisons that cause tissue hypoxia, and they are known as either simple, such as methane, or chemical, such as hydrogen cyanide (Chauhan et al., 2008). Agents like methane displaces oxygen from the air that is inspired, causing hypoxia. Chemicals like cyanide, on the other hand, cause disruption at a cellular level in oxygen transportation, which is referred to as

histotoxic hypoxia (White, 2002) (Figure 1.4). Symptoms include dizziness, confusion, and cardiorespiratory arrest. There are specific antidotes for exposure to blood agents such as nitrites and thiosulfate.

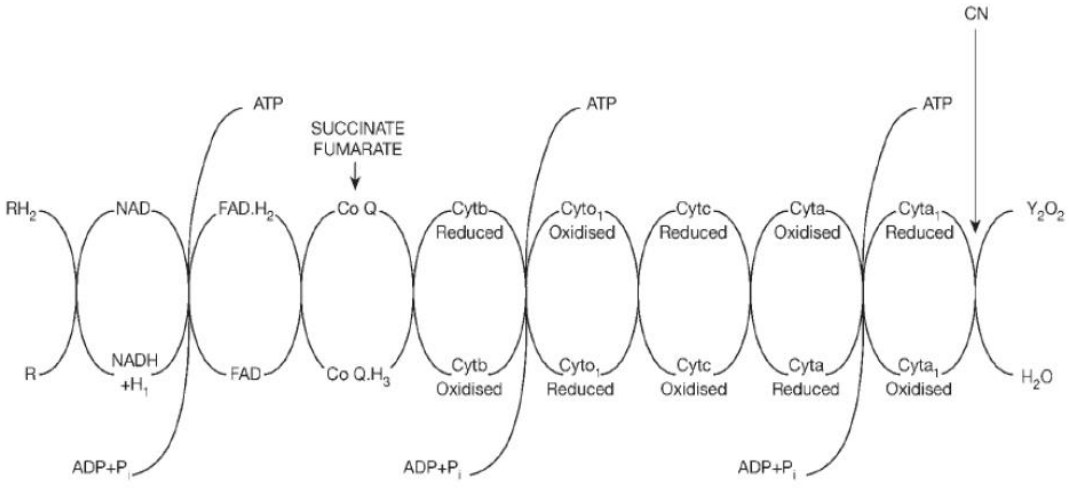

Figure 1.4 Cyanide (CN) inhibits cytochrome oxidase enzyme responsible for cellular respiration. Antidotes either physically remove CN from the enzyme or convert it to thiocyanate (Taken from Cummings, 2004).

Behaviorual agents are chemicals that debilitate physical and mental performance, such as tear gas, ketamin or LSD (White, 2002). Their effects include visual hallucinations, confusion, tachycardia and elevated blood pressure. Although not lethal, the purpose of using such chemicals is to disrupt the regular functioning of body physiology to create disturbance in enemy lines.

1.1.1.2 Biological Warfare Agents (BWAs)

Biological warfare is the intentional use of live microorganisms or toxins of biological origin in order to cause mortality or morbidity against an enemy. The most important difference between chemical and biological warfare agents is that CWAs tend to exert their effects immediately or in just a couple hours. BWAs, on the other hand, usually need incubation periods that can last for days, weeks or even months. Centers for Disease Control and Prevention (CDC, USA) divide BWAs in 3 categories (Table 1.2).

Table 1.2 Categorization of BWAs (Taken from Frischknecht, 2003)

| Crucial biological agents (Centers for Disease Control, Atlanta, Georgia, USA)

Disease	Pathogen	Abused[1]
Category A (major public health hazards)		
Anthrax	*Bacillus antracis* (B)	First World War Second World War Soviet Union, 1979 Japan, 1995 USA, 2001
Botulism	*Clostridium botulinum* (T)	–
Haemorrhagic fever	Marburg virus (V) Ebola virus (V) Arenaviruses (V)	Soviet bioweapons programme – –
Plague	*Yersinia pestis* (B)	Fourteenth-century Europe Second World War
Smallpox	*Variola major* (V)	Eighteenth-century N. America
Tularemia	*Francisella tularensis* (B)	Second World War
Category B (public health hazards)		
Brucellosis	*Brucella* (B)	–
Cholera	*Vibrio cholerae* (B)	Second World War
Encephalitis	Alphaviruses (V)	Second World War
Food poisoning	*Salmonella, Shigella* (B)	Second World War USA, 1990s
Glanders	*Burkholderia mallei* (B)	First World War Second World War
Psittacosis	*Chlamydia psittaci* (B)	–
Q fever	*Coxiella burnetti* (B)	–
Typhus	*Rickettsia prowazekii* (B)	Second World War
Various toxic syndromes	Various bacteria	Second World War

Category C includes emerging pathogens and pathogens that are made more pathogenic by genetic engineering, including hantavirus, Nipah virus, tick-borne encephalitis and haemorrhagic fever viruses, yellow fever virus and multidrug-resistant bacteria. [1]Does not include time and place of production, but only indicates where agents were applied and probably resulted in casualties, in war, in research or as a terror agent. B, bacterium; P, parasite; T, toxin; V, virus.

According to CDC, Category A BWAs are highly contagious and easily disseminated. These pathogens have the potential to cause high mortality rate, which in turn results in social disruption and panic, putting an excessive burden on healthcare system in specially asymmetric warfare. *Bacillus anthracis*, which has been used as a BWA in the past, is one of the most notorious bioweapons in this category (Figure 1.5). For some of the pathogens such as *Variola major*, viral agent that is responsible for smallpox disease with mortality rate between 20-60% if supportive treatment is received, or *Bacillus anthracis*, bacterial agent that causes anthrax with mortality rate 50% if early treatment is received, if not, 100% death rate from inhalational type, or Ebola virus giving rise to haemorrhagic fever with mortality rate between 60-90%, and depending on the virus species and adequate supportive care, death rate being as low as 20%, there are no curative treatments or approved vaccines yet, although such studies are still underway (Barquet & Domingo, 1997; Chambers et al., 2021; Kadanali & Karagoz, 2015). For bacterial agents, though, broad spectrum antibiotics can be used for curative treatment if received onset of infection (Thavaselvam & Vijayaraghavan, 2010).

Category B agents such as *Vibrio cholerae*, bacteria that is responsible for cholera disease, are also dangerous pathogens and can cause high morbidity rates, although their mortality rates are lower compared to Category A agents (Das & Kataria, 2010). However, BWAs in this category are still easy to disseminate to large masses. In fact, in 1930s, Japan built a secret biological weapon programme with various bacterial agents, one of them being *Vibrio cholerae.* During the Second World War, Japanese army contamined more than 1000 water wells in Chinese villages to test the effect of these agents on enemy population, and these biological attacks were reported to kill at least 30.000 people in 1947 (Frischknecht, 2003).

Category C agents are perhaps the most dangerous class of pathogens because they hold a potential to cause high mortality rates like those in Category A, and although for many of pathogens in Category A no curative treatment options are available, at least those agents are well studied. Pathogens placed in Category C, on the other hand, are easy to obtain and can also be genetically modified to alter their pathogenicity. In fact, such attempts have already been made by Soviet Union (Aken & Hammond, 2003). USSR's biological weapons programme produced 'invisible anthrax' by modifying the genome of *Bacillus anthracis* to give the pathogen new immunological properties. Nipah virus, for instance, is considered a Category C agent. This zoonotic pathogen, discovered in 1999 in Malaysia, caused a local outbreak in the region and infected 265 people, killing 105 of them, with 40% mortality rate (Mushtaq et al., 2006).

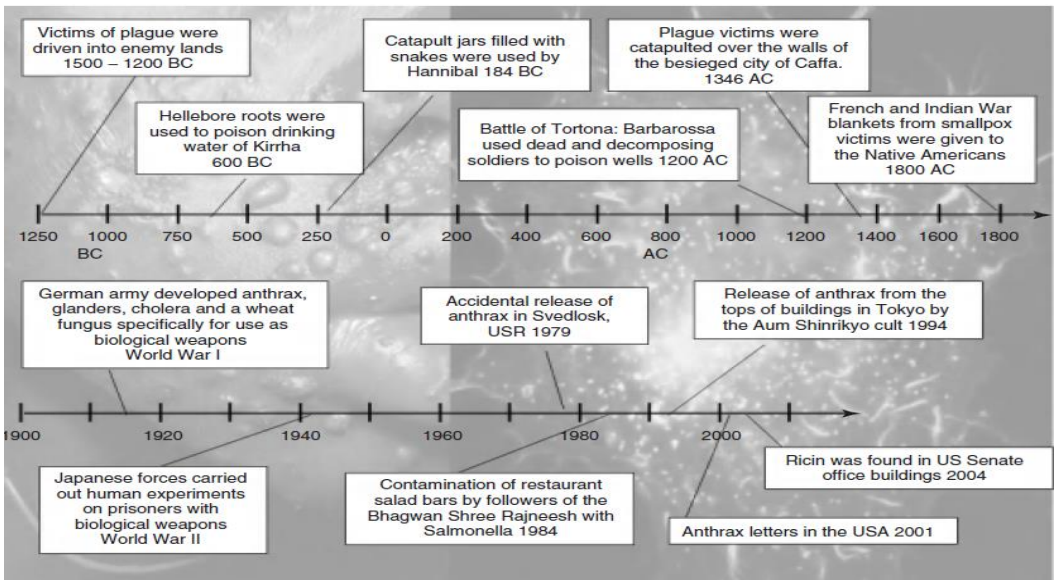

Figure 1.5 Examples of biological weapon deployments from history (Richardt et al., 2013)

Microorganisms need to possess certain characteristics to be considered as living weapons, or BWAs (Figure 1.6). Not all pathogens are prospective BWAs. For example, HIV causes a deadly infection. However, the virus cannot survive outside the body for long, which makes it, along with its route of infection, not eligible as a candidate to be used as a biological warfare agent (Moore, 1993).

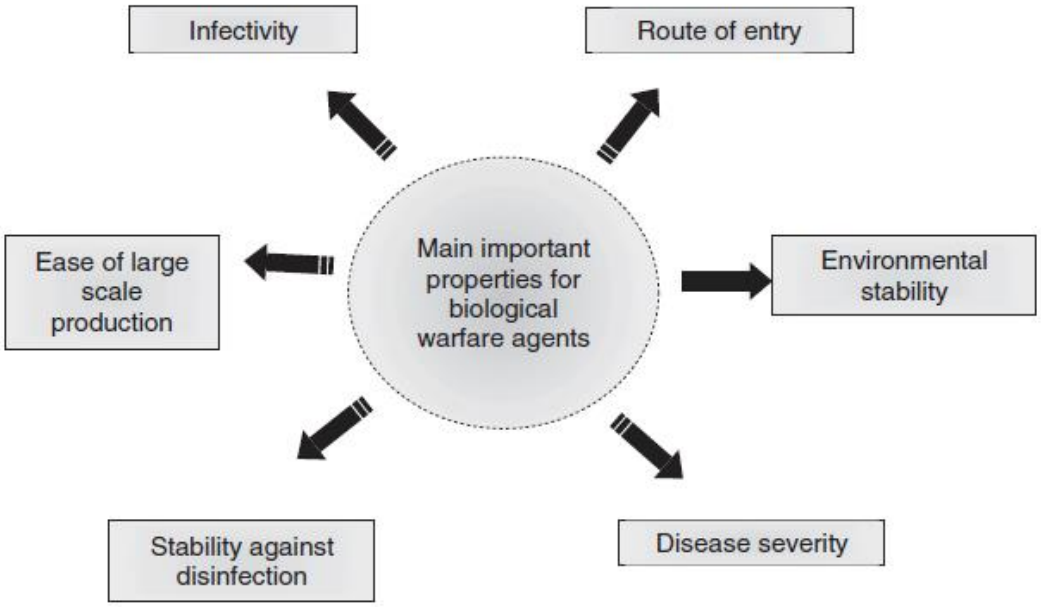

Figure 1.6 General characteristics of BWAs (Taken from Richardt et al., 2013)

1.1.1.3 Radiological and Nuclear Warfare Agents (RWAs-NWAs)

After the attack on Japan in 1945, nuclear weapons have become perhaps the most feared weapons of mass destruction. This is partly because a single warhead has the ability to cause destruction of an entire city (Richardt et al., 2013*).* Radioactivity refers to the process of emission of particles from nucleus as ionizing radiation. In that sense, nuclear weapons are actually subset of RWAs. The distinction between RWAs and NWAs is that RWAs can exist and be found naturally such as Uranium-238. On the contrary, NWAs are produced by nuclear fusion or fission reactions, and thus cannot occur naturally in the environment, such as Plutonium (Meulenbelt, 2018). Not dismissing the indications on human health, the actual purpose of using RWAs is not to inflict mass casualties, as opposed to nuclear weapons, but to cause economic loss and to create panic in public. So, RWAs are actually the weapons of choice for terrorist organizations to inflict disorder among people.

Even though radiological and nuclear weapons are considered to bring about the fear when even mentioned, their use in history shows that these agents of mass destruction cause only a small fraction of CBRN incident reported thus far (Table 1.3).

Table 1.3 Number of CBRN incidents reported (Cameron et al., 2000)

Type of Agent	Number of Incidents
BWAs	95
CWAs	65
NWAs	5
RWAs	5
Unknown	5

1.2 CBRN Decontamination

The most important difference between attacks using CBRN materials and conventional tactics is that aftermath of a CBRN incident, there is an additional burden of need for decontamination. This process refers to the neutralization or removal of CBRN agents from contaminated surfaces and also the victims (Kumar et al., 2010). Forms of contamination can be in solid, liquid, aerosol or vapor form, depending on the agent used.

1.2.1 Personnel Decontamination

1.2.1.1 Decontamination of Personnel for Chemical Warfare Agents

In the event of a CBRN incident, due to the nature of using such agents, first responders must be trained HAZMAT specialists. Many countries, including Turkey, also have special military units exclusively trained for combat against or in presence of CBRN agents. If, in a terrorist attack or a war, such chemical agents are used, there are certain criteria to consider. For instance, the type of CWAs used is important, as different categories of CWAs differ in their chemical properties (Table 1.4). Another factor is if contamination of personnel happens indoor or outdoor, as in outdoor environment, chemical agents would be diluted in air much quickly. In any case, if there are contaminated personnel, quick decontamination is of crucial medical importance.

When contaminated with nerve agents, first, rapid decontamination of external body should be performed. That is, if personel protective gear was worn beforehand, removal of such equipment would be enough to get rid of toxic chemicals. However, if there is contact with bare skin, then the contaminant should be removed as soon as possible, since nerve agents, although sometimes referred to as nerve gases, are mostly in liquid form, and quickly absorbed by the skin due to their lipohilic structure (Figueiredo et al., 2018).

Table 1.4 Chemical properties of CWAs (Taken from Talmage et al., 2007)

Principal chemical warfare agent degradation products with associated chronic toxicity guidelines

Agent/synonyms (CAS number)	Degradation process	Degradation product or impurity (CAS number)	Persistence[b]/ chemical–physical properties	Chronic toxicity values of degradation products[c]
Sulfur mustard (H, HD) (505-60-2)	Hydrolysis	Thiodiglycol (111-48-8)	Moderate nonvolatile miscible with water resistant to hydrolysis biodegradable	RfD: 400 μg/kg/day RfC: 469 μg/m^3
Lewisite (dichloro-(2-chlorovinyl)arsine) (541-25-3)	Hydrolysis, dehydration	Lewisite oxide[d] (3088-37-7)	High water-insoluble potential oxidation in soil	RfD: 0.1 μg/kg/day
VX (*O*-ethyl-*S*-(2-(diisopropylamino)ethyl) methylphophonothioate) (50782-69-9)	Hydrolysis	EA 2192[e] (73207-98-4)	Moderate low volatility high water solubility resistant to hydrolysis	RfC: 6×10^{-4} μg/kg/day RfC: 7×10^{-4} μg/m^3
	Hydrolysis	Ethyl methylphosphonic acid (EMPA) (1832-53-7)	Moderate low volatility water-soluble resistant to hydrolysis biodegradable[f]	RfD: 28 μg/kg/day RfC: 34 μg/m^3
	Hydrolysis	Methyl phosphonic acid (MPA) (993-13-5)	High low volatility resistant to photolysis resistant to hydrolysis high water solubility mobile in soils resistant to biodegradation	RfD: 20 μg/kg/day[g] RfC: 24 μg/m^3
GA (tabun; ethyl *N*,*N*-dimethylphosphoramidocyanidate) (77-81-6)	Hydrolysis	None of potential concern	—	—
GB (sarin; isopropyl methylphosphonofluoridate) (107-44-8)	Hydrolysis	Isopropyl methylphosphonic acid (IMPA) (1832-54-8)	High low vapor pressure water-soluble resistant to hydrolysis resistant to biodegradation	RfD: 100 μg/kg/day RfC: 110 μg/m^3
	Hydrolysis	Methyl phosphonic acid (MPA) (993-13-5)	High low volatility resistant to photolysis resistant to hydrolysis high water solubility mobile in soils resistant to biodegradation	RfD: 20 μg/kg/day[g] RfC: 24 μg/m^3
	Impurity	Diisopropyl methylphosphonate (DIMP) (1445-75-6)	High low volatility water-soluble resistant to hydrolysis slow biodegradation	RfD: 80 μg/kg/day[h]
GD (soman; pinacolyl methylphosphonofluoridate) (96-64-0)	Hydrolysis	Methyl phosphonic acid (MPA) (993-13-5)	High low volatility resistant to photolysis resistant to hydrolysis high water solubility mobile in soils resistant to biodegradation	RfD: 20 μg/kg/day RfC: 24 μg/m^3

For such instances, the US Army has used M291 Skin Decontamination Kit, which is basically absorbing the toxic liquid from the surface of the skin, and owing to the presence of its ion exchanger resin, partially decomposes nerve and blistering agents. In liquid form, RSDL (Reactive Skin Decontamination Lotion) is commonly used decontamination solution. Its active ingredient is 2,3-butanedione monoximate is known to neutralize nerve agents as well as T2 mycotoxin (Clarkson & Gordon, 2015) (Figure 1.7).

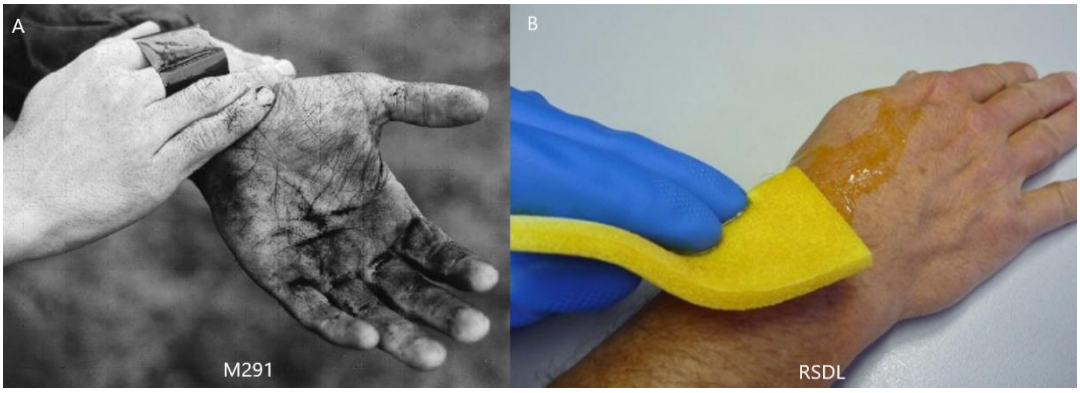

Figure 1.7 A) M291 Skin Decontamination product. B) Reactive Skin Decontamination Lotion (Taken from Clarkson & Gordon, 2015).

All in all, the main principle for decontamination of CWAs is to remove the toxic substances from surface of the body. Decontamination, as stated, can mean the removal of these agents from the skin or chemical neutralization of such agents.

1.2.1.2 Decontamination of Personnel for Biological Warfare Agents

For BWAs, decontamination and disinfection have two distinct meanings. When it comes to living weapons, such as *Variola major*, decontamination simply means removal and reduction of such pathogens. Disinfection, on the other hand, refers to the elimination of microbes (Richardt et al., 2013). Biological weapons can be used in many ways against both civilian population and military targets, however method of dispersion is not unlike chemical agents. In fact, using contaminated objects to spread infection has been used before. During the American civil war in 1863, native americans were given blankets contaminated with *Variola major*, viral agent responsible for smallpox (Barras & Greub, 2014). Although it is not clear if this attempt had caused an outbreak, such examples underly the importance of preparation against the worst of scenarios.

Contamination through objects is a possibility for many of the BWAs, however the most plausible method for dispersion of such agents would be in aerosol form. Dispersal of small droplets in the air can result in the infection of large numbers through inhalation. Nowadays, it is known that the ongoing Covid-19 pandemic is contracted through such a way.

If contamination occurs by a BWA, first, removing the clothing and cleaning the exposed skin with soap and 0.5% sodium hypochlorite are recommended mainly (Braue et al., 2011). This is partly because neither of these materials are hard to come by. However, for decontamination of biological agents from human skin, more specific formulas are needed.

1.2.1.3 Decontamination of Personnel for Radiological Warfare Agents

Radiological contamination poses a different kind of burden. Unlike decontamination of chemical and biological agents, radioactive materials cannot be deactivated. In other words, radioactive decay cannot be influenced. Because of that, the direct course of action for victims of such event is to physically remove the radioactive particles by washing the skin and wiping the contaminants off (Kaszeta, 2013).

1.2.2 Environmental Decontamination

1.2.2.1 Environmental Decontamination for Chemical Warfare Agents

Environmental decontamination is distinctly easier than personnel decontamination as many of the decontaminants used are highly corrosive to the skin. There are basically 3 methods of decontamination against CWAs; weathering, physical removal and neutralization (Richardt et al, 2013). Weathering simply means waiting out for CWAs to dissipate and degrade in outdoor environments on their own. This can be applied to contaminated vehicles and gears, or even for large sections of area. Physical removal refers to the mechanical ways such as using pressurized water to clean off contaminants from surfaces of materials. Neutralization is the use of certain solutions such as sodium hydroxide to break down CWAs.

1.2.2.2 Environmental Decontamination for Biological Warfare Agents

Biological contamination of equipment and places can be performed using a variety of decontamination solutions. These solutions are called biocides, and their

mechanism of action differs. They can be oxidizing and alkylating agents, protein denaturants or nucleic acid binding agents (Table 1.5). Although very effective, it is important to mention that these solutions are not applicable to human body.

Table 1.5 Antimicrobial effect of certain biocides (Taken from Richardt et al., 2013)

Chemical agent	Microbicidal activity[a]							Comment
	Bacteria				Viruses	Fungi		
	Spores	Gram positive	Gram negative	Myco-		Yeast	Mold	
Peracetic acid	+++	+++	+++	+++	+++	+++	+++	pH-optimum 2–3, fast reactivity
Na-hypochlorite	+++	+++	+++	+	+++	+	+	pH-optimum 4–6
Formaldehyde	+++	+++	+++	+++	+++	+	+	pH-optimum 4–9
Glutaraldehyde	+++	+++	+++	+++	+++	+	+	pH-optimum 2–3
Phenols	–	+++	+++	+++	+++	+	+	pH-optimum 2–4
Alcohols	–	+++	+++	+++	+++	+	+	–

[a] +++: high efficiency, +: moderate efficiency, –: no efficiency.

1.2.2.3 Environmental Decontamination for Radiological Warfare Agents

Environmental decontamination of radiological and nuclear warfare agents, in principle, is no different than that of personnel. As stated, radioactive particles cannot be deactivated from emitting radiation, and as such, decontamination efforts aim to remove these radioactive nuclides from equipments and vehicles by physical means (Figure 1.8). If a large area has become in constant irradiation by

contamination, then to cordone off the entire area may be the only feasible choice as it was done in Chernobyl site.

Alkyl polyglycosides, a class of surfactants, are generally preferred for such decontamination procedures because of their low toxicity and biodegradability (Richardt et al., 2013).

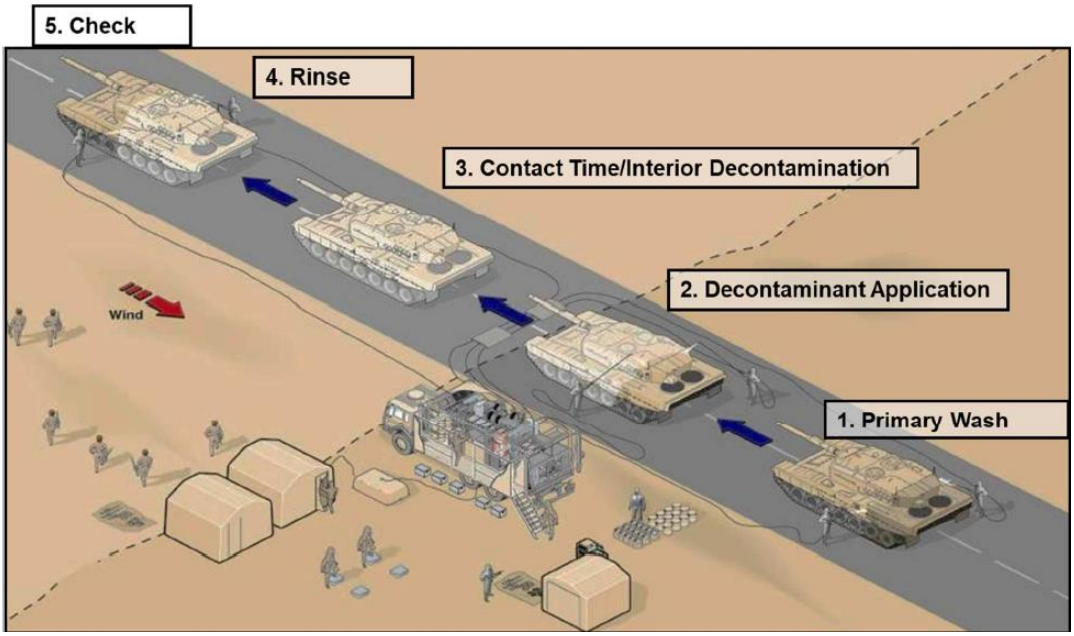

Figure 1.8 Representation of military R & N decontamination site (Richardt et al., 2013)

1.3 Zeolite Powder

The word zeolite comes from greek words ‘zeo’, meaning boil, and ‘lithos’, meaning stone, as in ‘boiling stone’ since most zeolite structures are generated as a result of volcanic activity (Moshoeshoe et al., 2017). Previous studies have reported that zeolites, crystalline microporous aluminosilicate powder, could be used as

antimicrobials as well as to be gas absorber, food additive, odor control agent, and water disinfectant owing to their large surface area, structural stability at high temperatures and chemically neutral basic structure (Özogul et al., 2018).

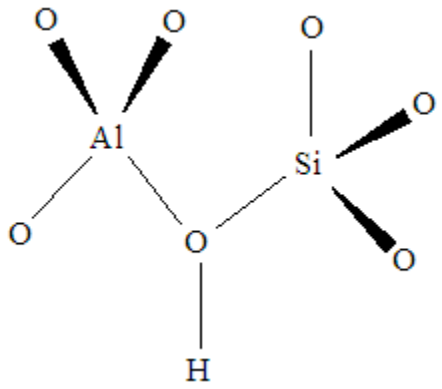

Figure 1.9 Aluminosilicate structure of zeolite (Taken from Moshoeshoe et al, 2017)

1.3.1 Antimicrobial Effect

For antimicrobial effect, these zeolite materials can be loaded with inorganic metal ions such as zinc, silver, copper, mercury or lead through ion exchange to give them antibacterial properties (Yuan and Qian, 2016). Zeolite compound that has been used in this study contains silver particles. Furthemore, the decon formulation contains zincoxide and titanium dioxide nanoparticles.

It has been reported that silver nanoparticles (AgNPs), due to their high surface area, have stronger antibacterial effect compared to bulk silver metal (Panayotova et al, 2018). Considering that there is a growing concern for antibiotic resistance that has

become a worlwide problem in recent years (Aslam et al, 2018), use of silver nanoparticles are once again being brought under spotlight since silver and silver ions are known to have high antibacterial effectiveness and low toxicity for human body (Zhang et al, 2009). Silver has already been used in many aspects of our daily life such as in cosmetics, detergents, shoes, food containers and various medical devices. It should also be mentioned that AgNPs also have inhibitory properties for viruses and fungi (Dutta and Wang, 2019).

Zinc oxide (ZnO) is another compound that has been shown to have antimicrobial properties. It was reported ZnO nanoparticles induce antibacterial effect for both Gram positive and Gram negative bacteria as well as bacterial spores (Tiwari et al, 2018). Although the exact mechanism of its antibacterial effect is not clear, it is thought that hydrogen peroxide generation could be one reason (Xie at al., 2011). Another factor proposed is that ZnO binding to bacterial surface because of electrostatic interactions could account for the antibacterial effect as well (Stoimenov et al., 2002).

Titanium dioxide (TiO_2) nanoparticles have also been reported to induce inhibitory effect on bacteria by oxidation (Ahmed et al., 2020). Another study stated that TiO_2 nanoparticles can cause decomposition of organic compounds through the release of hydroxyl radicals and superoxide ions following exposure to non-lethal UV light (Jesline et al., 2014).

1.3.2 Use of Protamine for Antimicrobial Effect

Protamine sulfate is a polycationic peptide that has been known and used for its action to antagonize the anticoagulative effect of heparin in cardiac surgeries (Boer et al., 2018). Its antimicrobial properties against variety of bacteria and yeast were also recognized and tested (Kim et al., 2014). It has been reported that protamine

introduction to *P.aeruginosa* has caused the condensation of cytoplasm. Enlargement in periplasmic space was also observed and damage in cell membrane, as in small perforations, caused cell lysis (Aziz et al., 2019).

1.4 Blood Coagulation

Injury to the integrity of skin causes bleeding. Superficial injuries ruptures capillaries and as a result, coagulation cascade is activated (Adams & Bird, 2009). Blood coagulates and bleeding stops (Figure 1.9). However, deep lacerations may injure larger vessels, causing a serious bleed out that needs to be stopped by outside intervention. The main route of infection for biological agents is through inhalation and to some degree, gastrointestinal tract by consumption. Intact skin would not allow microorganism to penetrate into the system. However, during CBRN incidents, losing the integrity of the skin puts additional risk of systemic contamination through such openings.

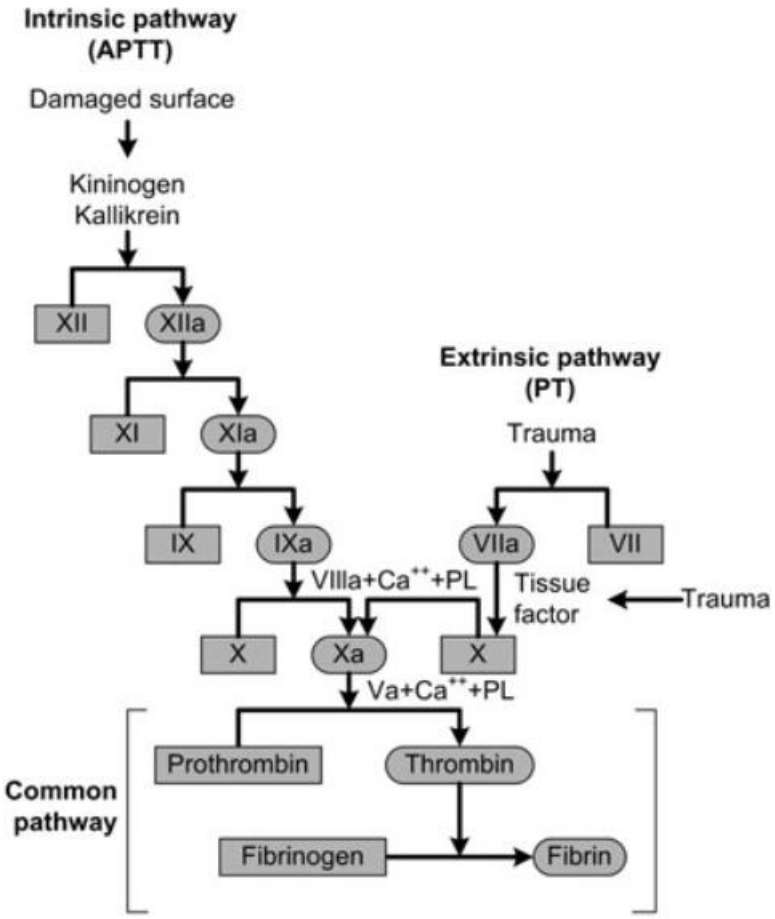

Figure 1.10 Physiology of blood coagulation cascade (Taken from Adams & Bird, 2009)

1.4.1 Use of Zeolite for Blood Coagulation

Hemostatic action of zeolite has been known and is already being made use of in some commercially available combat gauzes such as Quikclot. It has been reported that zeolite initiates exothermic process resulting in adsorption of water (Eryilmaz et al., 2009). This process was found to physically aggregate erythrocytes and platelets, and as a result, clumping of these cells act as a barrier to bleeding.

Figure 1.11 Commercially available zeolite based combat gauze.

1.4.2 Use of Chitosan, Alginate, Gelatin and Protamine for Blood Coagulation

Different hemostatic products are also in use and their potential to stop bleeding was reported. However, it is important to state that all commercially available gauzes found today are for superficial injuries, not for arterial bleedings. Their potential to initiate blood coagulation is limited.

Chitosan is a linear amino polysaccharide and deacetylated form of chitin (Lovskaya et al., 2020). These natural biorenewable biopolymers are acquired from crustacean shells. Both chitosan and chitin have biomedical applications such as tissue engineering, drug delivery systems and hemostatic gauze preparations (Berretta et al., 2020). Since chitosan is positively charged, when applied on bleeding wounds, chitosan impregnated gauzes adheres to erythrocytes, clumping them together and creating a barrier to stop bleeding (Pogorielov et al., 2015).

Alginate is another natural biopolymer of polysaccharide extracted from brown algae (Lee & Mooney, 2012). Hemostatic effect is due to adhesive properties of alginate-impregnated gauzes to wounds. In addition, sodium salt of alginate has also been reported to have high rate of water absorption, thereby retaining blood cells at the wound site (Huang et al., 2019).

Gelatin, derived from collagen, is comprised largely of natural polypeptide (85-92%) and mineral salts (Duconseille et al., 2015). This biopolymer is largely used in pharmaceutical industry to produce hard capsules. Although there is still controversy about the effectiveness of gelatin as a hemostatic agent, it was shown that gelatin assembles a meshwork at the wound site, thereby enclosing and trapping blood cells

and facilitating clot formation. Also, after absorption of blood, gelatin tends to expand in volume, acting to form tamponade (Natalie et al., 2018).

Although, as stated, protamine is used for its action to reverse the anticoagulative effect of heparin in surgeries, since it is positively charged, its adhesive properties on EDTA-treated blood was investigated in this study.

1.5 Aim of the Study

CBRN Defence is a multidisciplinary science that still has not received the necessary attention that this field needs, considering the multitude of the potential consequences of weapons of mass destruction. As an important part of defense, decontamination of such materials should be performed with utmost efficiency and haste. As most of the commercially available products in this field are liquid solutions, there is a need for different forms of decon materials for different contingincies. As such, we wanted to test the antimicrobial effect of a decon powder formulation of zeolite structure in powder form on Gram positive and Gram negative bacteria. Furthermore, since bacterial endospores are very resilient structures that can withstand harsh conditions such as high temperatures and antibiotic treatment, we wanted to test whether our new formula has inhibitory effect on spore viability as well. In addition to these, we also investigated the coagulatory effect of the decon powder formulation on blood in case skin integrity is compromised as a result of CBRN incidents.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

The chemicals used in this study included tryptone, yeast extract, sodium chloride, agar, protamine, sodium alginate, gelatin, chitosan, Schaeffer and Fulton spore stain solutions A and B, and decontamination powder which were acquired from Sigma-Aldrich, AppliChem, and Nanobiz Technology Inc.

Decontamination powder provided by Nanobiz Technology Inc. is of mainly zeolite structure containing silver nanoparticles (AgNPs), titanium dioxide and zinc oxide in its formulation.

2.2 Growth Conditions of *Escherichia coli*

E. coli ATCC 25922 strain (biosafety level 1) was used in the experiments as Gram-negative bacteria. The cells were grown using Luria Bertani broth, which is made up of 10 g bacto-tryptone, 10 g NaCl, 5g yeast extract (pH 7.5) for 1 L medium. To prepare LB agar plates, in addition to these, 15 g agar was added. Sterilization was conducted using autoclave. From stock cultures (preserved at -80°C), 30 µL *E. coli*

sample was spread on agar plate using glass spreader and left for incubation at 37°C for 18 hours. The next day, single colony was selected using loop from the plate and transferred to 50 mL conical tube containing 10 mL LB and incubated at 37 °C with shaking at 150 rpm for 18 hours

2.3 Growth Conditions of *Bacillus subtilis*

Bacillus subtilis PY79 strain (biosafety level 1) was used in the experiments as Gram-positive bacteria. Like *E. coli*, LB and LB agar media was used to grow *B. subtilis*. From the stock culture, 40 µL *B. subtilis* sample was spread on agar plate and incubated at 37°C for 18h. The next day, single colony was selected and transferred to 10 mL LB containing 50 mL conical tubes at 37 °C with shaking at 150 rpm for 18 hours.

2.4 Sporulation

For the sporulation of *B. subtilis*, special agar medium was used. First, from the stock cultures, using LBA, bacteria were grown overnight. Then, selecting and transferring single colony, they were grown in LB broth. Afterwards, 100 µL from the broth were taken and spread on sporulation agar media. To prepare the media, first, LB broth was prepared. Then, 1g/L KCl, 0.12 g/L $MgSO_4.7H_20$, and 15 g/L agar were added and autoclaved. After sterilization, when the temperature of the mixture dropped to 45°C, 1M $CaNO_3.4H_20$ (1ml/L), 0.0182M $MnCl_2.4H_20$ (1ml/L), and 1mM

$FeSO_4.7H_20$ (1ml/L) solutions were prepared and added using filter-sterilization. Then, the solution was poured into agar plates. After waiting for 30 minutes for agar to solidify completely, 100 µL *B.subtilis* sample from overnight growth was transferred and spread on agar plates. Then, inoculated plates were left for incubation at 37°C for 6 days.

2.5 Staining and Visualization of Spores

In order to confirm the presence of spores, Malachite green staining was performed (Oktari et al, 2017). Following the incubation period at 37°C for 6 days, 750 µL sterile 0.9% NaCl solution was distributed on agar plate, and using sterilized (autoclaved) cotton swabs on one third section of the plate, samples were taken and transferred to 2 mL sterile microcentrifuge tubes containing 1mL 0.9% NaCl solution. Then, the 2 mL microcentrifuge tubes prepared were placed in heat block dry bath (Techne Dri-Block DB2D) at 80°C for 30 minutes in order to get rid of any vegetative cells and external contamination. Following this, all the samples in the tubes were transferred to sterile 50 mL conical tube. Afterwards, 30 µL of spore solution was transferred to a slide. After giving it 3 to 4 minutes to air dry completely at room temperature, heat fixation was performed by passing the slide over flame 3 to 4 times carefully. Then, when the smear was ready, malachite green stain was transferred on the slide covering the middle section where spores were distributed and waited for 60 seconds. Afterwards, the slide was passed through the flame for 3 to 5 minutes, adding malachite green solution to prevent it from drying out in the process. Then, the slide was washed with dH_20, and this was followed by the addition of safranin stain. After 60 seconds, the slide was washed again using dH_20 and was first examined using compound light microscope (Leica) to visually confirm the

presence of the spores, then images were acquired via Floid Cell Imaging System (Figure 2.1).

Figure 2.1 EVOS Floid Cell Imaging Station used to visualize B.subtilis spores

2.6 Application of Decontamination Powder on Bacterial Samples

From 10 mL LB broth left for incubation overnight at 37°C at 150 rpm, 30 µL (1:50 diluted) samples were transferred on sterile (autoclaved) glass covers. Various doses of the powder (from 50 mg to 750 mg) were then placed on 30 µL bacterial samples on glass covers so that the powder could fully absorb the sample. Different time intervals, starting with 15 minutes, 30 minutes, 60 minutes, 120 minutes and 180 minutes were tried in order to decide the shortest duration of time for decon powder

to exert its antimicrobial effect. Then, the samples, with glass covers, are placed in 10 mL LB containing sterile 50 mL conical tubes. Also, in addition to these intervals, one sample is always transferred to the conical tubes without waiting, and this sample is referred to as 0-hour sample in this study to test the immediate action of the decon powder.

2.7 Dilution

Following overnight incubation of *E.coli* and *B.subtilis* with decon powder, dilution was performed before inoculation on agar plates similar to that shown in Figure 2.2. Preliminary studies showed that 10^{-5} dilution rate yielded less than 1000 colonies in control groups, which was countable. To achieve 10^{-5} dilution rate, serial dilution was performed. Starting with 10^{-1} dilution from incubated (overnight) broth, 100ul of bacterial sample was mixed with 900 µL sterile LB using sterile 2 mL microcentrifuge tubes. Following this, 2 consecutive 10^{-2} dilutions were performed by taking 10 µL from the previously 10^{-1} diluted sample and mixing it with 990 µL of sterile LB. In the end, dilution rate of 10^{-5} samples were acquired.

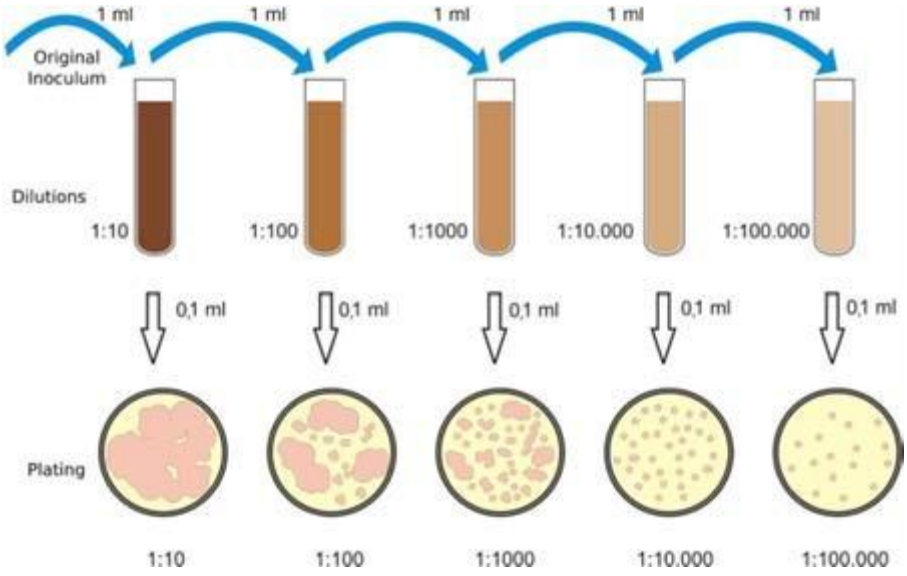

Figure 2.2 Visual representation of serial dilution method (Taken from Alves and Cruvinel, 2016)

2.8 Colony Forming Unit (CFU) Count

CFU amount was determined by counting individual colonies on agar plates that were left for overnight growth. When exact number is known on agar plate, using the below formula (Figure 2.3), CFU count was performed in control and decon powder treated groups.

$$\text{CFU/ml} = \frac{(\text{No. of colonies x Total dilution factor})}{\text{Volume of culture plated in ml}}$$

Figure 2.3 general formula for the calculation of CFU per mL

2.9 Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration is defined as the lowest dose of an antimicrobial agent inhibiting the visible growth of microorganism (Wiegand et al, 2008). Determination of MIC was performed through the use of varying concentrations of decon powder. Lowest dose used was 50 mg, whereas highest dose was 750 mg. Also, for comparison, protamine was also used in 10 mg and 50 mg doses. In addition to these, mixture of decon powder and protamine was also utilized in order to see whether there is synergistic interaction between them. Doses applied to *E.coli*, *B.subtilis* and *B.subtilis* spores are summarized in Tables 2.1- 2.3.

For each treatment, experiments were designed in 3 sets (triplicates), and for *B. subtilis spores*, critical dose groups were designed in 9 sets.

For each set, 7 different agar plates were used, and one of them was a negative control in order to show that without decon powder, bacterial growth will not be affected. To see the immediate antibacterial action, on 30 µL bacterial samples, different amount of decon powder and protamine were added and without waiting, placed in 10 mL containing conical tubes. This sample was referred to as 0-h. To test the reduction in colony numbers, another sample was kept waiting for 15 minutes, which was referred to as 15-min sample. Effects of decon powder on bacterial samples were also investigated for 30 minutes, 60 minutes, 120 minutes and 180 minutes separately.

Table 2.1 Dosages of decon powder and protamine Applied on E.coli.

Decon Powder	Decon Powder+Protamine	Protamine
100mg	50mg+50mg	10mg
250mg	90mg+10mg	50mg
500mg	200mg+50mg	
750mg		

Table 2.2 Dosages of decon powder applied on B.subtilis.

Decon Powder
50mg
100mg
250mg
500mg
750mg

Table 2.3 Dosages of decon powder and protamine applied on B.subtilis spores

Decon Powder	Decon Powder + Protamine	Protamine
100mg	90mg + 10mg	10mg
250mg	200mg + 50mg	
500mg		

2.10 Preparations of Gauzes and Solutions for Coagulation using Whole Blood Sample

For coagulation experiments with whole blood samples, different chemicals known to induce *in vitro* erythrocyte aggregation were used (Sivakumar et al, 2017). Sodium alginate, gelatin, protamine and chitosan solutions were prepared in different concentrations. In addition to these, decon powder was also used to observe whether it has coagulative properties. The procedure used here was modified from Chou et al, 2012.

For preparing alginate solutions, 1,5 g and 3 g alginate were dissolved in 100 mL dH_2O using magnetic stirrer (WiseStir MSH-20A), so that 1.5% and 3% alginate solutions could be acquired. The same procedure was also used to prepare 1.5% and 3% gelatin and protamine solutions. For chitosan solutions, however, 2% acetic acid solution was used to dissolve it, since chitosan would only dissolve in acid aqueous mixtures.

After the solutions were ready, 1x1 cm standart gauzes that are frequently used during surgical practices were cut, and soaked in these solutions for 60 seconds. After that, they were placed in incubation chamber at 40°C to dry for 2 hours in glass petri dishes. Following this, on solution-impregnated gauzes, 100 µL EDTA-treated whole blood samples were added. For decon powder, however, since it cannot dissolve in any solution, 500 mg samples were distributed homogenically onto gauzes (Figure 2.4). Furthermore, from all solutions (alginate, gelatin, protamine and chitosan) 20 mL volume was transferred to a different beaker to get a mixture of all.

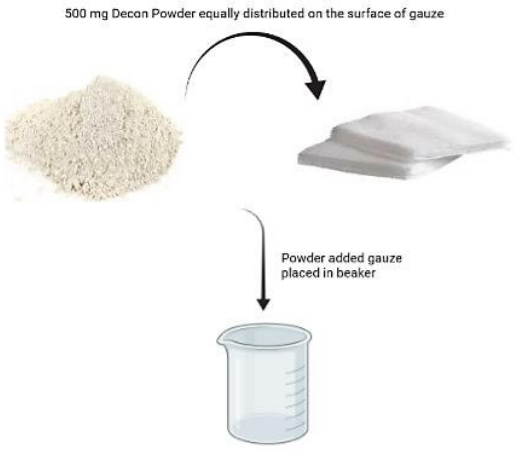

Figure 2.4 Preparation of decon powder gauze.

At this stage, 2 sets of experiments were prepared with the same concentration of solution-impregnated gauzes. A minute was given for whole blood introduced gauzes to soak 100 µL blood. Later on, in beakers containing blood introduced gauzes, 20 mL dH_2O was carefully added (Figure 2.4). Once dH_2O was added, all beakers were placed on platform shaker (Heidolph Unimax 1010) at 100 rpm for 60 seconds in order to test their ability to withhold the blood in presence of physical disturbance. As reference, 100 µL whole blood was added in 20 mL dH_2O. 100 µL whole blood was also used on standard gauze without any chemical treatment to be used as a negative control. Two sets of spectrophotometric cuvettes were prepared for each experiment (Figure 2.5)

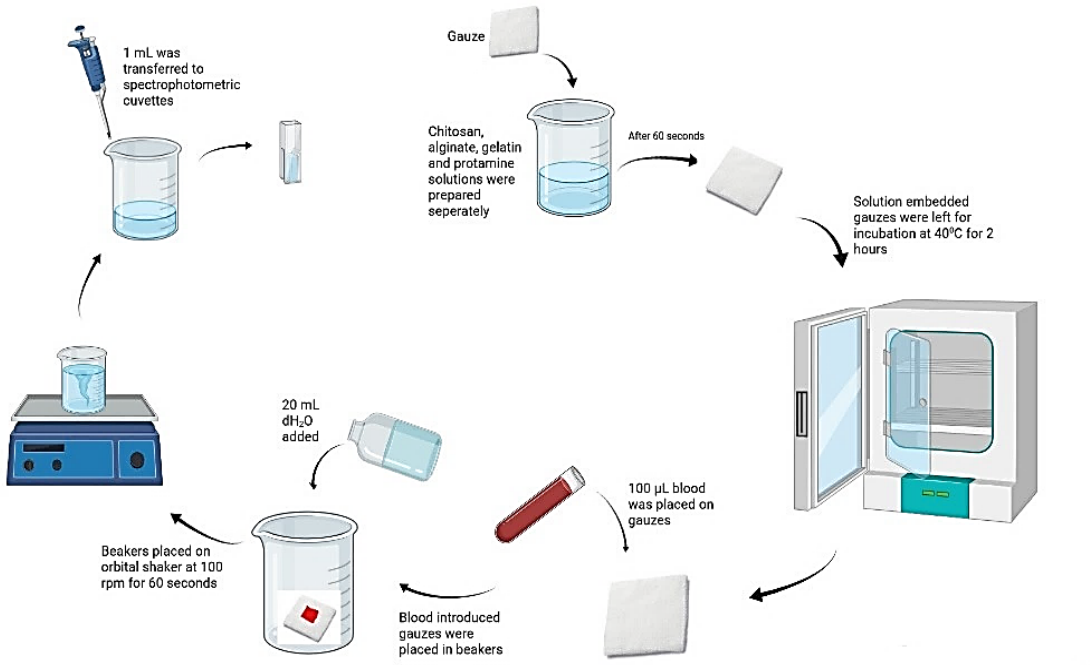

Figure 2.5 Procedure used to perform spectrophotometric analysis.

Because the decon powder would not dissolve, it creates turbidity within beaker when dH_2O is added, making an accurate spectrophotometric reading difficult. To avoid that, excluding the decon powder treated gauze, from all other beakers containing solution impregnated gauzes with blood, 1 mL samples were prepared before centrifugation in order to test if centrifuge that is performed causes reduction in hemoglobin concentration, thereby giving out false positive readings.

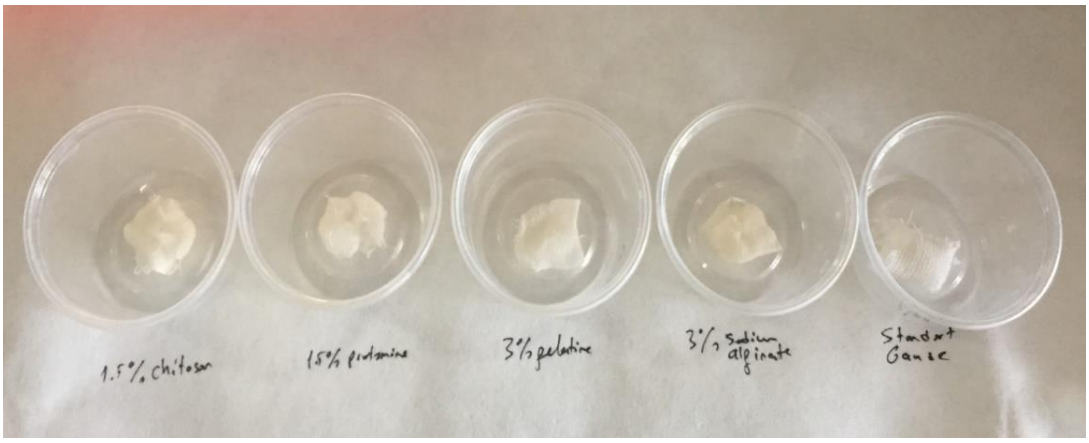

Figure 2.6 1x1cm gauzes after incubated at 40°C for 2 hours.

2.11 Spectrophotometric and Statistical Analysis

From every beaker, upon completion of abovementioned steps, 1 mL of sample was transferred to spectrophotometric cuvettes (Figure 2.7). At 540 nm, using dH_2O as blank, and 100 µL whole blood distributed in 20 mL dH_2O (without using a gauze) as reference, readings (Thermo Scientific Multiskan GO Microplate Spectrophotometer) were performed.

Higher OD values would suggest higher concentrations of hemoglobin from erythrocytes, which means that gauzes released some of the blood after dH_2O was added on them. High degree of release would indicate that, specially for solution saturated gauzes, blood was not trapped or withheld, meaning that blood coagulation property of such gauzes are low. Smaller OD values, on the other hand, would suggest that there is lower hemoglobin concentration in beakers from which spectrophotometric sample was taken, indicating that amount of hemoglobin released from the gauzes to dH_2O was lower, so the gauze could withhold the blood by clumping erythrocytes together.

In order to show that decon powder possesses coagulative properties in addition to antibacterial effect, mean OD values of samples of standard gauze and decon powder were calculated and two-sample t-test was performed.

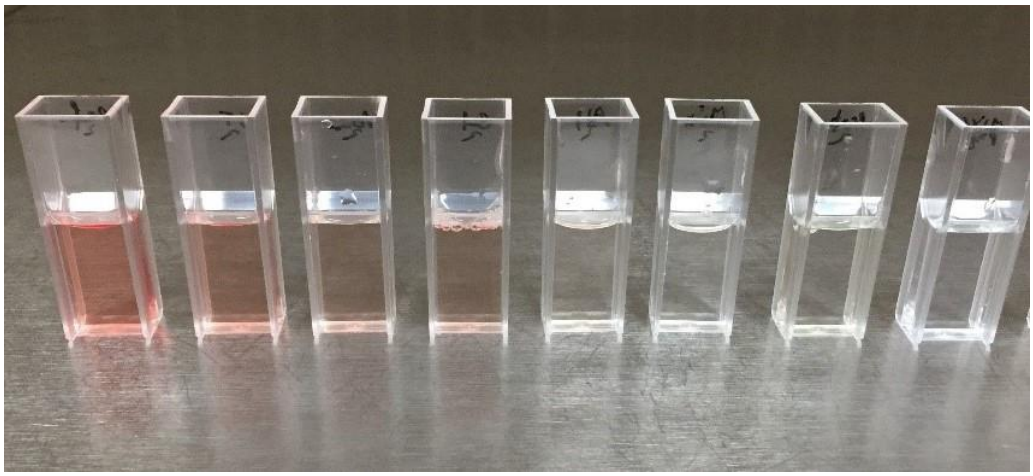

Figure 2.7 Spectrophotometric cuvettes containing (from left to right) reference (whole blood), standart gauze, 500mg powder, 3% gelatin, 1.5% alginate, mixture, 3% protamine, and 1.5% chitosan, respectively.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization Studies for Dilution

The purpose of serial dilution is to be able to track back the overall concentration of CFU amount in samples by counting the individual colonies on plates (Ben-David, 2014). Thus, bacterial colonies on agar plates should be in countable amount, that is, there should be less than 1000 colonies on a plate to be counted accurately. To find the correct dilution rate for this study, serial dilution method was used. Starting with control (no dilution), 10^{-4}, 10^{-5} and 10^{-6} dilution rates were tried for both *E. coli* and *B. subtilis* using 30 µL volume, as these samples would be placed on a cover slip, the volume should be as small as possible to prevent spillage over the edge of the cover slips. Using 30 µL *E. coli* on agar plates, 10^{-4} dilution yielded over 1000 colonies, 10^{-5} dilution gave 361 colonies, and 10^{-6} dilution showed only 64 colonies (Figure 3.1). Using the same volume of *B.subtilis*, again 10^{-4} dilution yielded over 1000 colonies, 10^{-5} dilution gave 217 colonies, and 10^{-6} dilution revealed only 19 colonies (Figure 3.2). Then, to show that 30 µL volume is suitable to be used with 10^{-5} dilution rate, along with 100 µL and 50 µL samples, plates were inoculated. It was observed that 100 µL volume yielded over 1000 colonies, 50 µL volume exhibited 936 colonies, and 30 µL volume gave 374 colonies for *E.coli* (Figure 3.3).

Then, the same procedure was repeated for *B.subtilis*, and found that for 100 µL, there were 504 colonies; for 50 µL, there were 244 colonies; and for 30 µL, 156 colonies appeared (Figure 3.4). In order to keep consistency in procedures, same dilution rate and volume were selected for both *E.coli* and *B.subtilis* as 30 µL volume and 10^{-5} dilution rate.

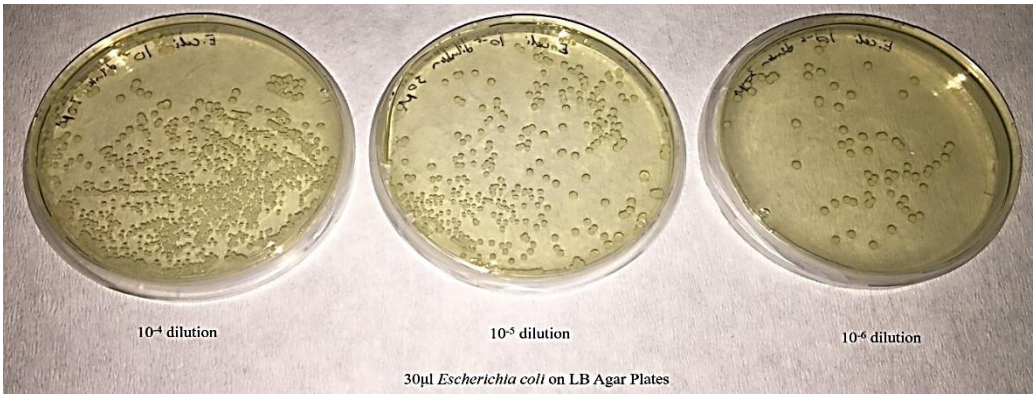

Figure 3.1 E. coli spread on agar plates with different dilution rates.

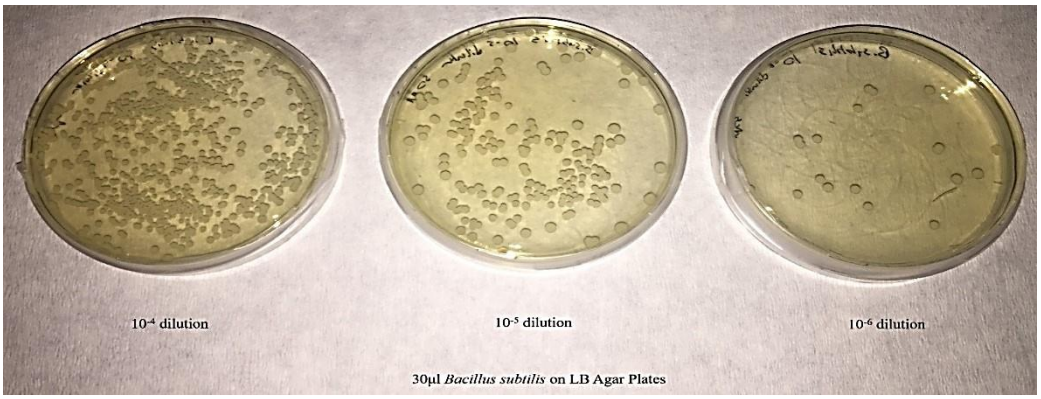

Figure 3.2 B. subtilis spread on agar plates with different dilution rates.

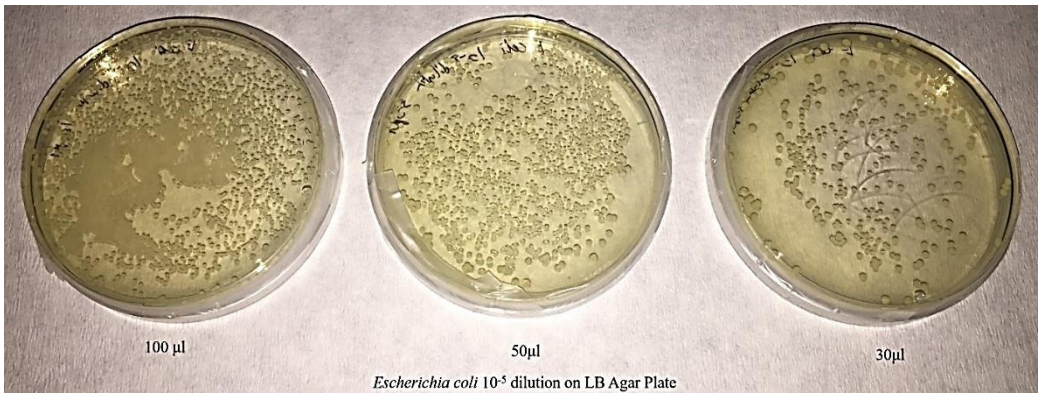

Figure 3.3 E.coli spread on agar plates using different volumes.

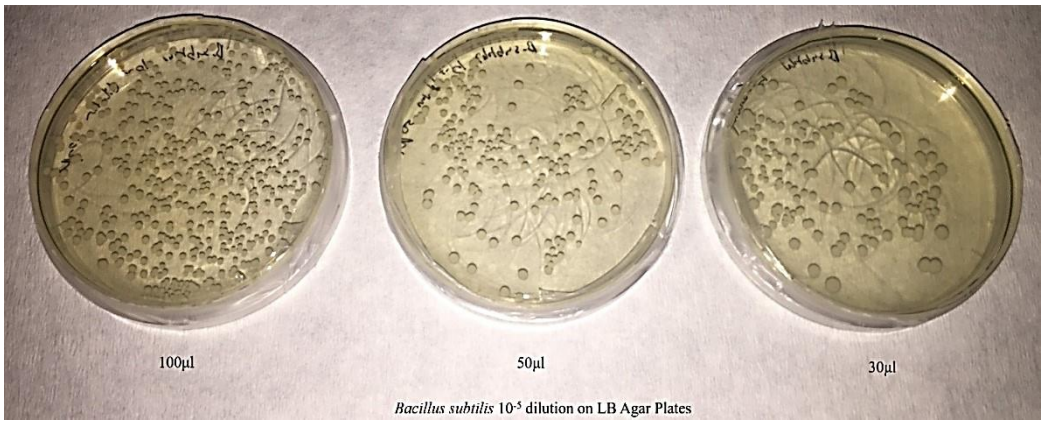

Figure 3.4 B.subtilis spread on agar plates using different volumes.

3.2 Antimicrobial Effect of Zeolite Powder

Antimicrobial effect of decon (zeolite) powder was investigated on two species of bacteria, *Escherichia coli* and *Bacillus subtilis.* Sporulation of *B. subtilis* was also performed and antimicrobial effect of the decon powder was tested on bacterial endospores as well.

In addition to the use of decon powder, protamine sulfate was also tested alone and in mixture with decon powder in order to observe its antimicrobial effect and to see whether there is synergistic interaction between the two antimicrobials when used together against Gram positive and Gram-negative bacteria.

Using different amounts of decon powder and different time intervals, experiments were designed and conducted to determine MIC values.

MIC is described as the lowest dosage of an antimicrobial agent inhibiting the visible growth of microorganisms (Wiegand et al, 2008). However, if there is visible growth of bacteria as distinct colonies on agar plates, by calculating the reduction (in percentage) in the number of colonies with respect to control groups, different MIC values can be assigned (Zhang et al, 2017). For instance, such commonly used values are MIC_{50} and MIC_{90}, meaning that whatever dosage was utilized, there is a 50% and 90% reduction in CFU number, respectively. MIC alone refers to the fact that there is no visible bacterial growth.

Decon powder used here does not dissolve in any liquid. Its antimicrobial effect comes from absorbing the bacterial sample directly owing to its zeolite form and because of this, mg/mL unit was not used, only the amount in milligram was mentioned.

In figures, only the first set of plates were represented along with graphs.

3.2.1 Antimicrobial Effect of Zeolite Powder on *E. coli*

Escherichia coli is a Gram negative, non-spore forming, usually motile, rod-shaped bacterium from *Enterobacteriaceae* family. As an important part of normal, healthy microflora, this bacterium lives in lower intestinal tract of warm-blooded animals including humans, and through feces, they are discharged into the environment (Hur et al, 2017). However, *E. coli* is known to be the most common cause of acute urinary tract infections, urinary track sepsis along with acute enteritis, diarrhea, neonatal meningitis and abscesses in many organs (Percival and Williams, 2014). *E. coli*, in this study, was chosen as a model bacterial organism for Gram negative pathogens, since cell wall structure of bacteria, for both Gram negative and Gram positive species, is an important factor for pathogenicity (Dörr et al, 2019). The strain ATCC 25922, which has been featured in this study, is a commonly used model strain for antimicrobial susceptibility testing. This strain is nonpathogenic and cannot produce verotoxin that causes hemolytic uremic syndrome by Enterohemorrhagic *E. coli* (Lingwood, 2020).

To test MIC values for *E. coli*, starting with 750 mg decon powder, the dosage has been gradually reduced to monitor the status of visible colony development on agar plates as observed in the following figures.

As seen in Figure 3.5, application of 750 mg decon powder directly on *E.coli* had an immediate action for reducing the number of colonies. The reduction in mean colony numbers was calculated to be 98.53% (Table 3.1). For 15-min samples, reduction in percentage increased to be 99.70%. There was no visible colony development starting with 30-min samples.

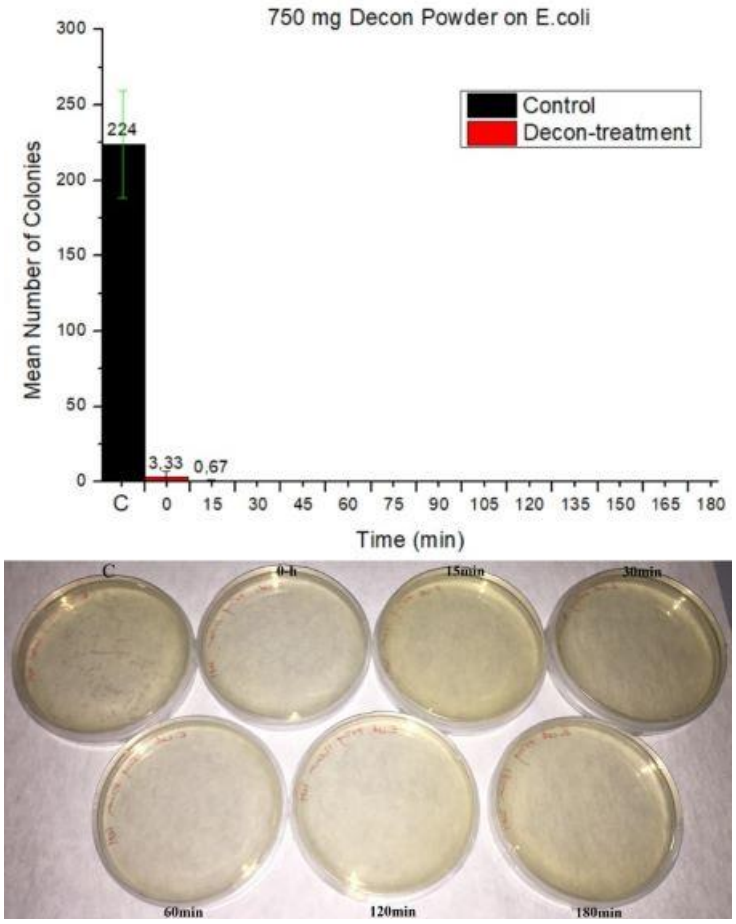

Figure 3.5 Mean number of CFU of 3 sets of E.coli treated with 750 mg decon powder.

When the dosage was reduced to 500 mg, a similar result compared to 750 mg decon powder was obtained (Figure 3.6). Even though there were only single colonies on 2 out of 3 15-min plates with 750 mg decon powder treated *E.coli*, no colony development was observed with 500 mg decon powder application after 15 minutes. 500 mg decon powder was also seen to have immediate antibacterial action with 98.65% reduction in 0-h plates (Table 3.1).

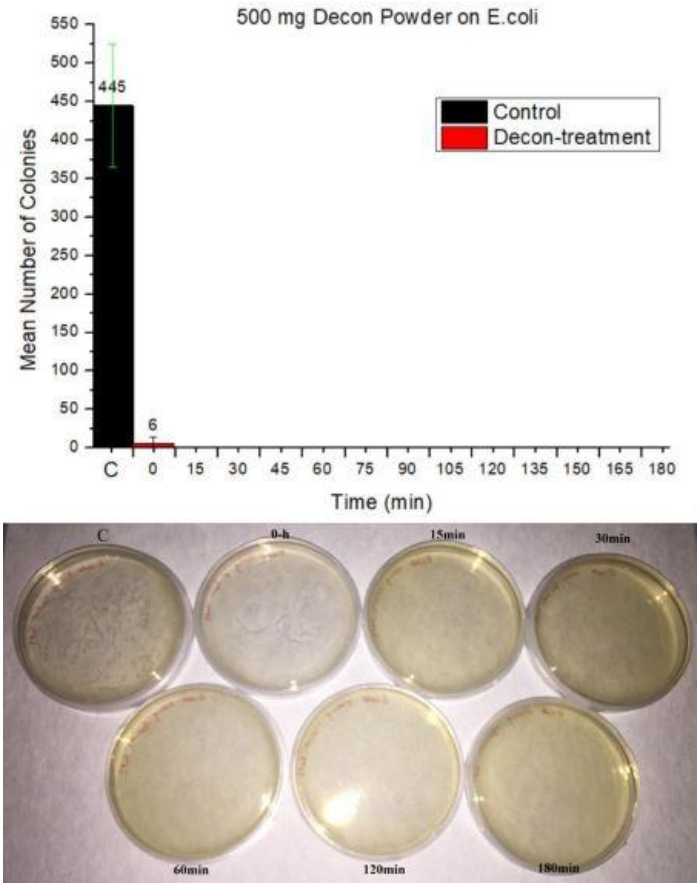

Figure 3.6 Mean number of CFU of 3 sets of E.coli treated with 500 mg decon powder.

When 250 mg decon powder was applied on *E.coli*, there was no visible colony development starting with 30-min samples (Figure 3.7). Mean number of colonies for 0-h samples was in fact observed to be higher than that of control group, indicating that 250 mg powder had no immediate antibacterial effect on *E.coli* . However, application of 100 mg decon powder reduced colonies by 98.26% for 15-min plates, and if given another 15 minutes, meaning with 30-min plates, the reduction in colony numbers increased to be 99.77% (Table 3.1). Similar to 250 mg decon powder application, 100 mg decon powder had no immediate antibacterial effect.

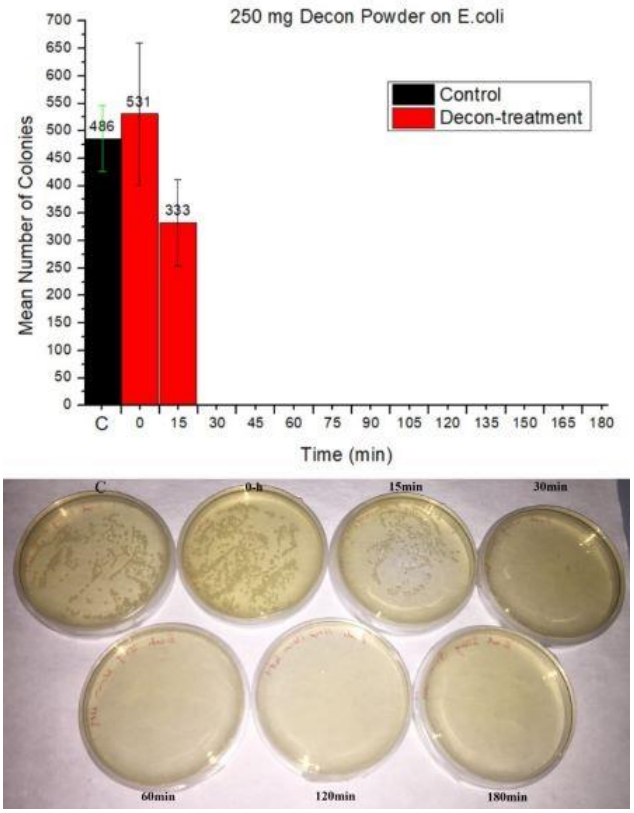

Figure 3.7 Mean number of CFU of 3 sets of E.coli treated with 250 mg decon powder.

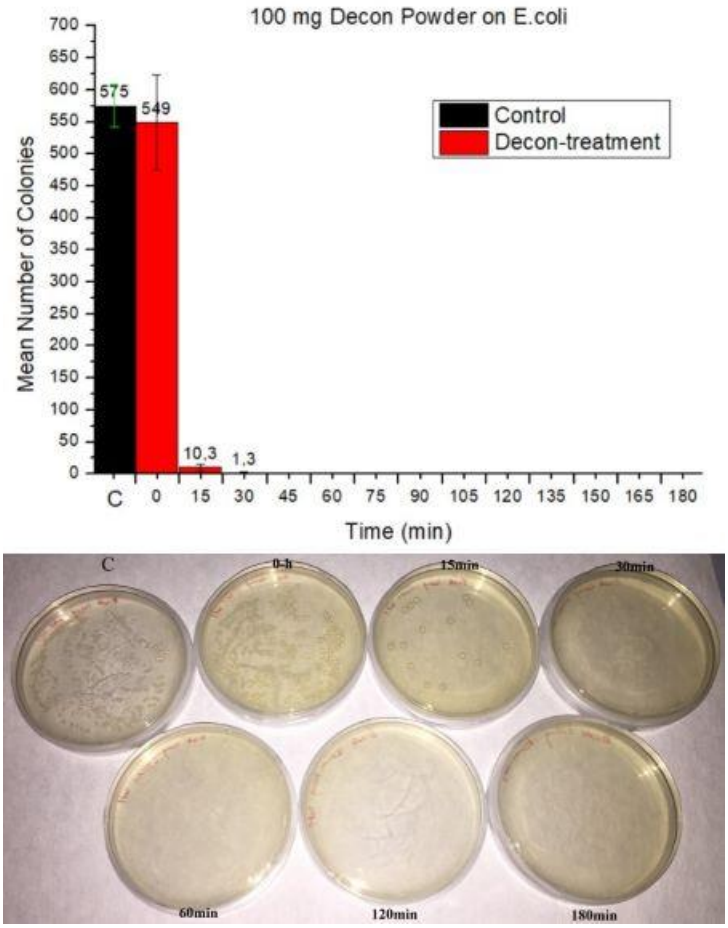

Figure 3.8 Mean number of CFU of 3 sets of E.coli treated with 100 mg decon powder.

Table 3.1 Mean CFU ± standart deviation, and percentage decrease (%) with respect to control groups for E.coli treated with decon powder.

E.coli	Control	0-h	15-min	30-min	60-min	120-min	180-min
750 mg	224 ±35,7	3,33 ±4,04 (98.53%)	0,67 ±0,57 (99.70%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)
500 mg	445 ± 79,5	6 ± 7,81 (98.65%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)
250 mg	486 ±60,14	531 ±129,5 (no decrease)	333 ±78,36 (31.48%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)
100 mg	575 ±33,72	549 ±74,27 (4.5%)	10,3 ±5.03 (98.26%)	1,3 ±1.52 (99.77%)	0 (100%)	0 (100%)	0 (100%)

Calculating the mean of all control plates, which is 433 colonies, 1,4 x 10^9 CFU/mL *E.coli* were treated with different concentrations of decon powder. According to Table 3.1, MIC value for *E.coli* can be stated as 500 mg decon powder for 0-h samples taking the standard deviation into account. This means that in order to see immediate antibacterial action on 30 µL *E.coli* (1,4 x 10^9 CFU/mL), at least 500 mg decon powder should be applied. 250 mg and 100 mg decon powder had no antibacterial effect in 0-h plates. Even though 98.26% reduction in colony number was observed for 100 mg decon powder with 15-min samples, there were no significant reduction when 250 mg decon powder was used. This can be explained by the fact that ingredients in powder responsible for antibacterial action may not

have been homogenically distributed while being transferred from the master batch to be used on *E.coli* as smaller dosage (100 mg) caused significant reduction in CFU amount. MIC value for 30-min plates was observed to be 100 mg, meaning that when 100 mg decon powder was used, in 30 minutes, there would not be visible growth.

3.2.2 Antimicrobial Effect of Zeolite Powder with Protamine on *E. coli*

The results shown thus far only used decon powder on *E. coli*. However, another natural peptide compound known to have antibacterial properties was also tested. Protamine was used alone in two different dosages (1 mg/mL and 5 mg/mL) and also used in conjunction with decon powder to test if there are any synergistic interactions between them on bacterial viability. In figures below, the results of mixing decon powder and protamine in different concentrations are shown.

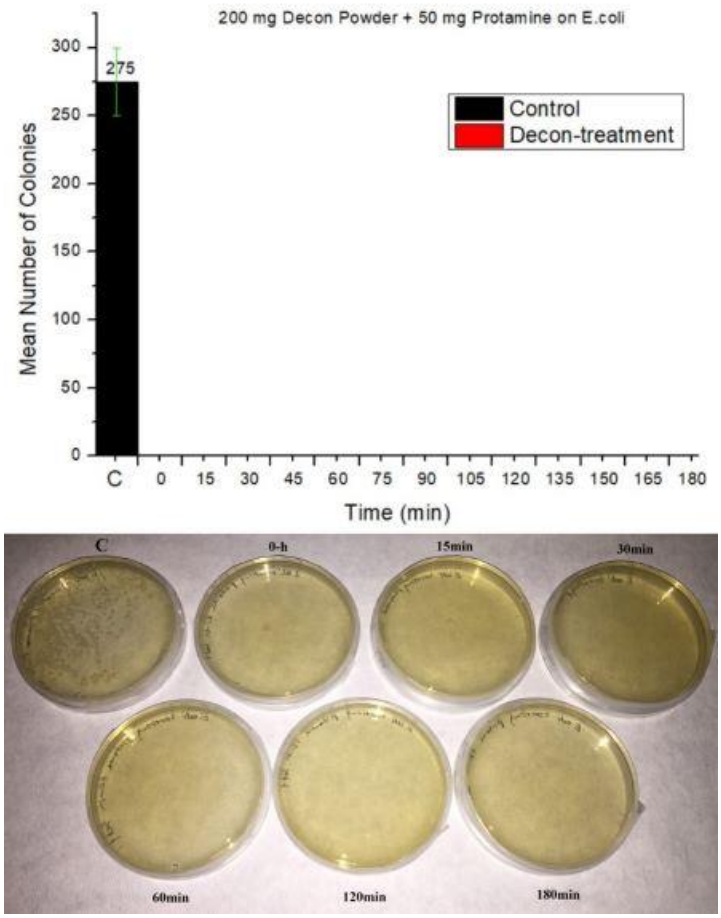

Figure 3.9 Mean number of CFU of 3 sets of E.coli treated with 200 mg decon powder mixed with 50 mg protamine.

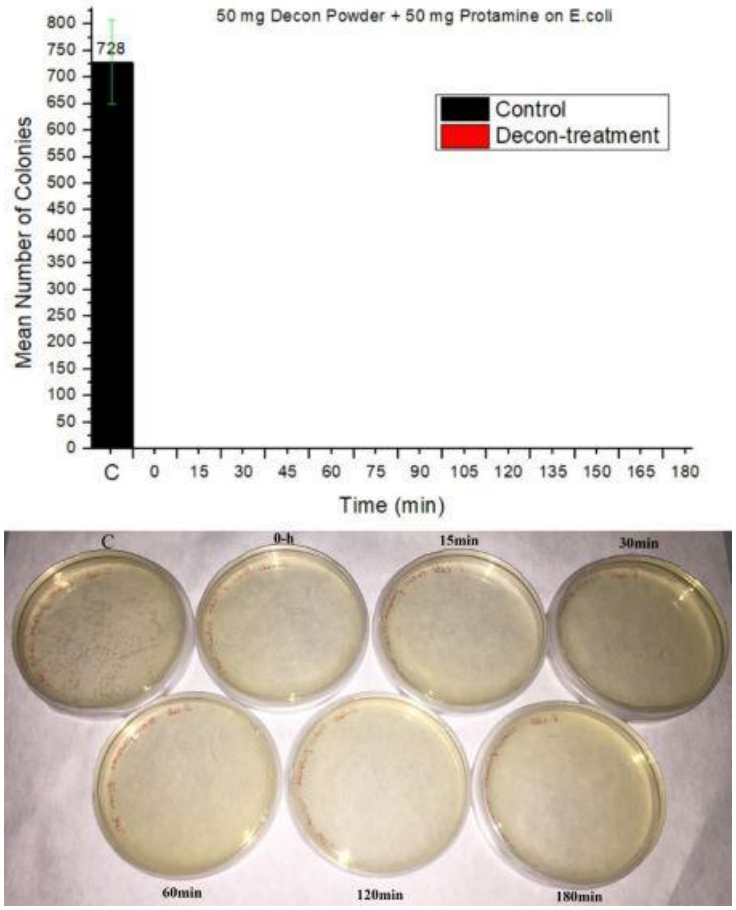

Figure 3.10 Mean number of CFU of 3 sets of E.coli treated with the mixture of 50 mg decon powder and 50 mg protamine.

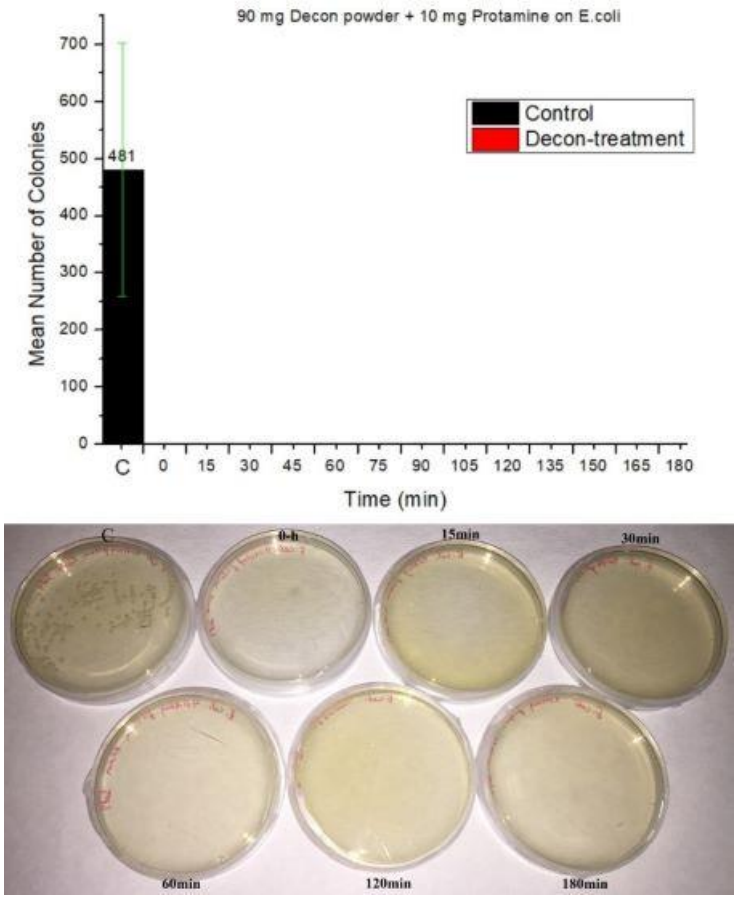

Figure 3.11 Mean number of CFU of 3 sets of E.coli treated with the mixture of 90 mg decon powder and 10 mg protamine.

The effect on bacterial viability of the mixture of decon powder and protamine with varying concentrations was investigated by gradually reducing and adjusting the dosages of both compounds (Figures 3.9, 3.10 and 3.11 and Table 3.2). It can be seen that no *E.coli* growth was present in any of the experimental setups.

Table 3.2 Mean CFU ± standart deviation, and percentage decrease (%) with respect to control groups for E.coli treated with decon powder mixed with protamine.

E.coli	Control	0-h	15-min	30-min	60-min	120-min	180-min
200 mg decon powder + 50 mg protamine	275 ±24,56	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)
50 mg decon powder + 50mg protamine	728 ±79,27	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)
90 mg decon powder + 10 mg protamine	481±221,51	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)

3.2.3 Antimicrobial Effect of Protamine on *E. coli*

In order to conclude that the absence of growth is due to synergistic interaction between decon powder and protamine, antibacterial effect of 50 mg and 10 mg protamine alone was also investigated. From Figures 3.12 and 3.13, it can be observed that starting with 0-hour plates, there is no visible *E.coli* growth. So, antibacterial effect of the mixture of 200 mg decon powder and 50 mg protamine is not necessarily coming from the synergistic interactions since 50 mg protamine alone

can also induce the same antibacterial effect. However, 10 mg protamine could only reduce 90.12% of colonies in 0-h plates, and although negligible considering the standart deviation, several colony developments were also observed with 15-min and 30-min plates (Table 3.3).

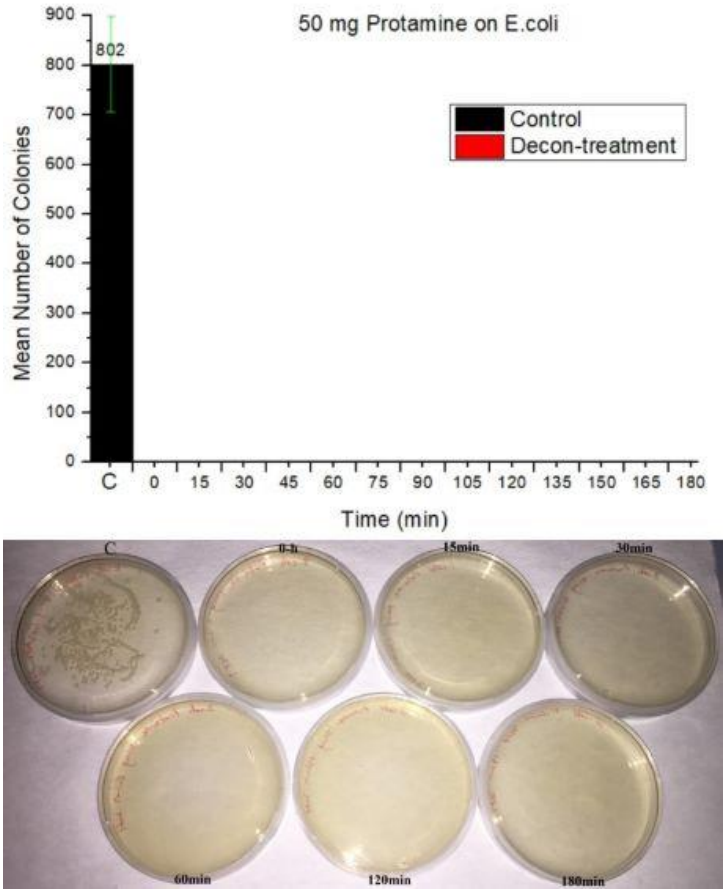

Figure 3.12 Mean number of CFU of 3 sets of E.coli treated with 50 mg protamine.

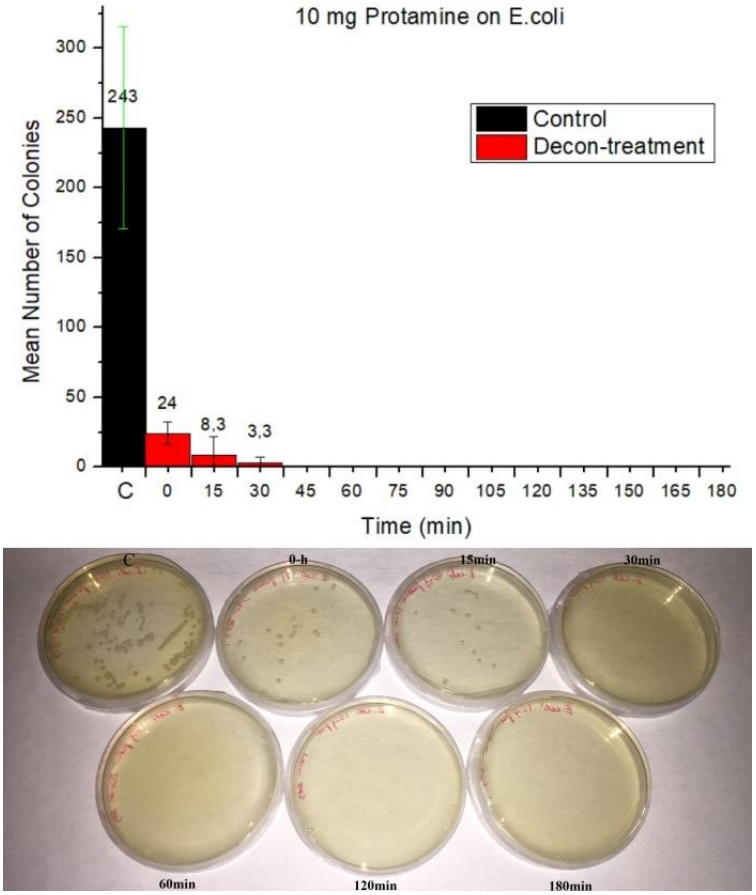

Figure 3.13 Mean number of CFU of 3 sets of E.coli treated with 10 mg protamine.

Table 3.3 Mean CFU ± standart deviation, and percentage decrease (%) with respect to control groups for E.coli treated with decon powder mixed with protamine.

E.coli	Control	0-h	15-min	30-min	60-min	120-min	180-min
50 mg protamine	802 ±96,02	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)
10mg protamine	243 ±72,38	24 ± 8,08 (90.12%)	8,3 ± 13,58 (96.71%)	3,3 ±4,16 (98.64%)	0 (100%)	0 (100%)	0 (100%)

Investigating the Figures 3.8, 3.11 and 3.13, we can state that mixing 90 mg decon powder with 10 mg protamine would show increased antibacterial activity when 100 mg decon powder or 10 mg protamine alone were applied. This in fact suggests a synergistic interaction between the two compounds.

The results so far, for *E.coli*, showed that minimum amount of dose to prevent the visible growth of bacteria with immediate action (0-hour plates) is the combination of 90 mg decon powder with 10 mg protamine. 500 mg decon powder alone could also induce immediate antibacterial effect. Results also showed that 50 mg protamine prevented the visible growth of colonies for 0-h plates, however since the purpose of this study is to test primarily the antimicrobial activity of decon powder in context of CBRN Defence, 100 mg mixture of decon powder and protamine is considered to be the lowest dose here.

3.2.4 Antimicrobial Effect of Zeolite Powder on *B. subtilis*

Bacillus subtilis is an aerobic, gram positive, spore forming, rod shaped soil bacterium that is commonly used as model organism for its ability to produce endospores for extreme environmental conditions such as heat, desiccation, nutrient limitation or in presence of toxic elements and also used for investigation of genetic transformation (Hartig and Jahn, 2012). *Bacillus subtilis* and *Bacillus anthracis* which has been used as a biological weapon that causes deadly anthrax belong to the same genus, and both can produce endospores (Driks, 2009). In this study, to test the antibacterial properties of decon powder against biological weapons, as a model organism for *B. anthracis, B. subtilis* was utilized.

In vegetative state, antibacterial effect of decon powder on *B.subtilis* was investigated using the same procedure applied for *E.coli*. Results have shown that as high as 750 mg decon powder and as low as 50 mg decon powder prevented the visible growth of bacteria on agar plates with immediate action. Because decon powder alone was effective against *B.subtilis* cells, protamine alone or in mixture with decon powder were not tried.

Except for control groups, there was no visible growth with any of the plates using 750 mg decon powder on *B.subtilis* as seen in Figure 3.14.

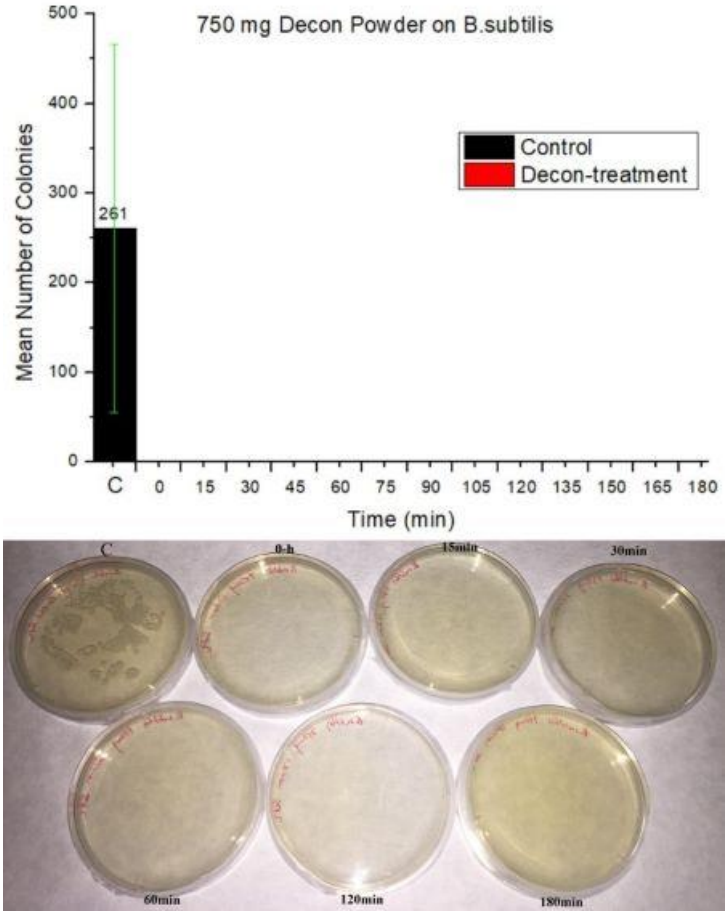

Figure 3.14 Mean number of CFU of 3 sets of B.subtilis treated with 750 mg decon powder.

Next, 500 mg decon powder was applied on *B.subtilis* samples. Apart from control plates, there was no visible growth with the sole exception of a single colony on 60 min plate in the first set of the experiment (Figure 3.15).

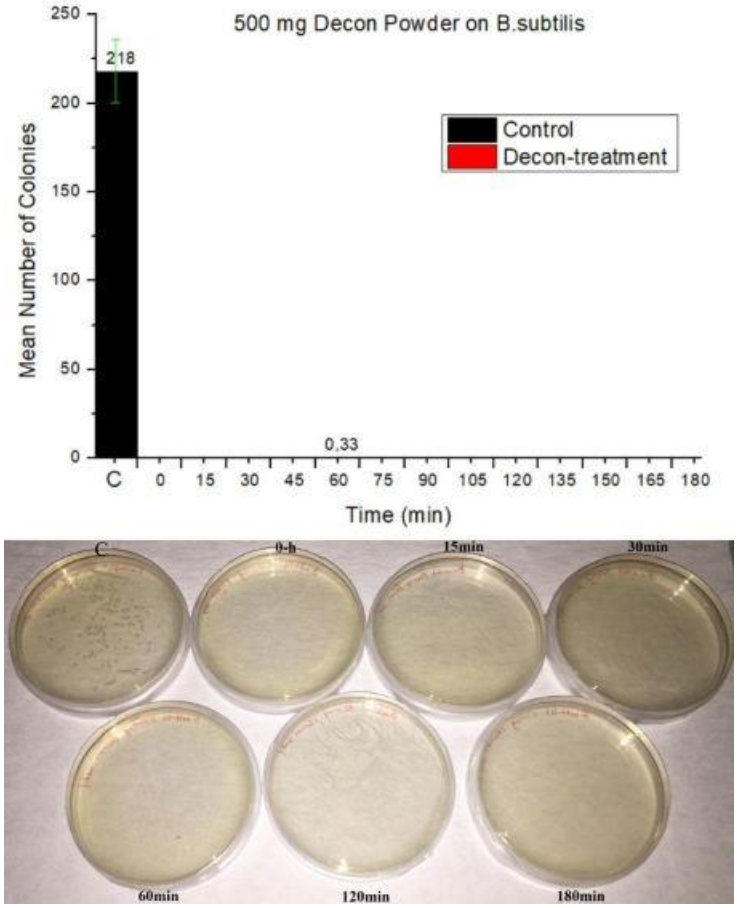

Figure 3.15 Mean number of CFU of 3 sets of B.subtilis treated with 500 mg decon powder.

When 250 mg decon powder was applied on *B.subtilis*, there was no visible growth for any of the plates except in the first set, only 180-min plate yielded 25 colonies, which can be attributed to external contamination since with 5 different dosages of decon powder, only one plate has shown as many colonies (25) as observed in Figure 3.16.

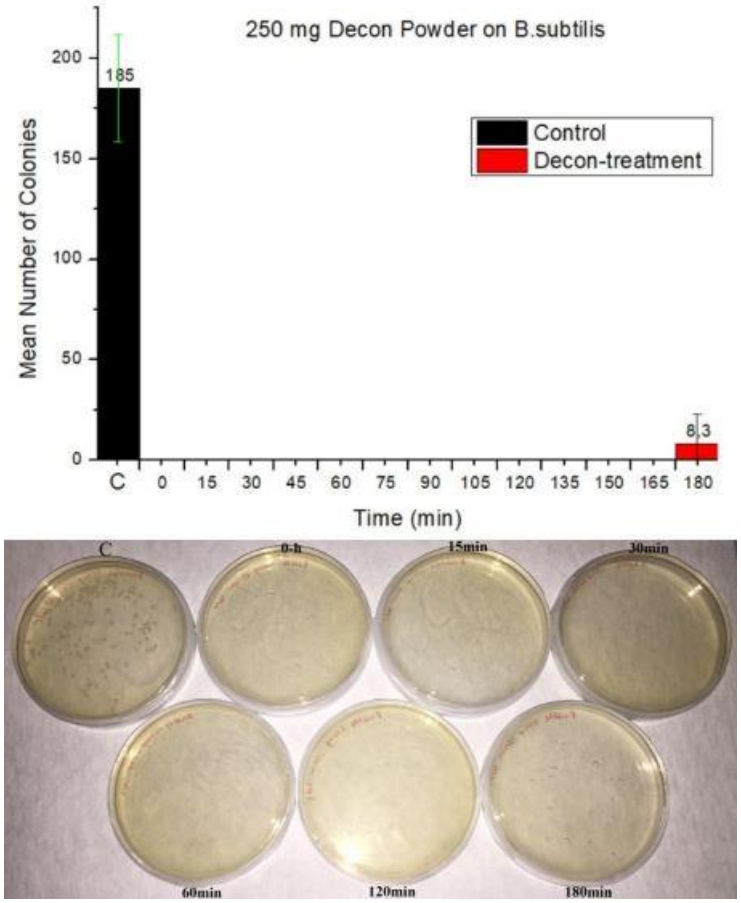

Figure 3.16 Mean number of CFU of 3 sets of B.subtilis treated with 250 mg decon powder.

We kept reducing the dose to 100 mg decon powder, and except for control plates, no visible growth was observed (Figure 3.17).

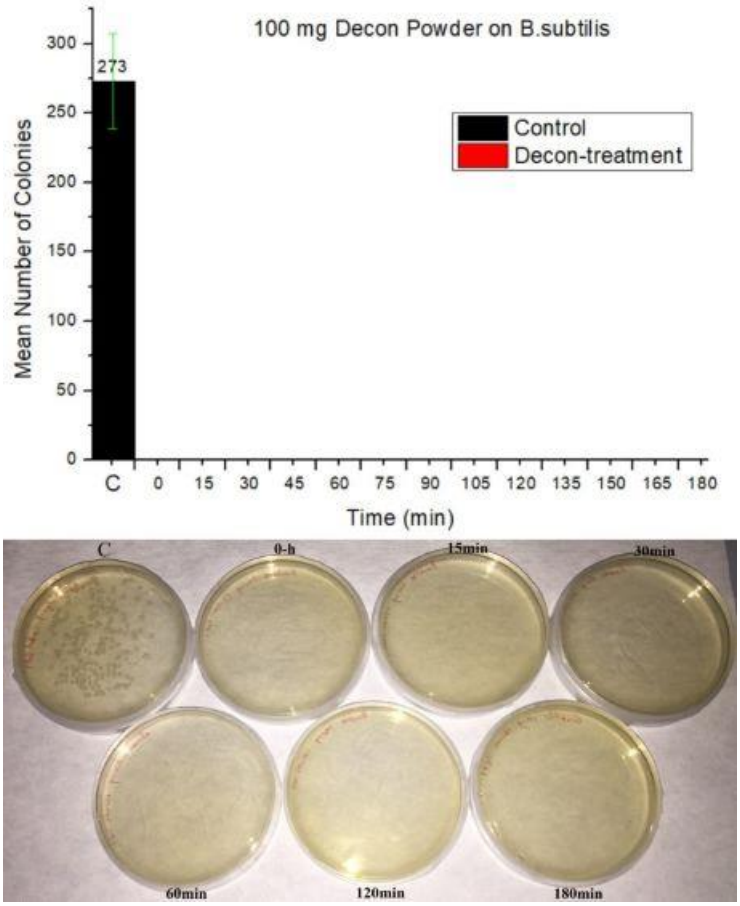

Figure 3.17 Mean number of CFU of 3 sets of B.subtilis treated with 100 mg decon powder.

Lastly, the dosage was decreased to 50 mg decon powder, and again, except for control groups, there was no visible growth of *B.subtilis* cells (Figure 3.18).

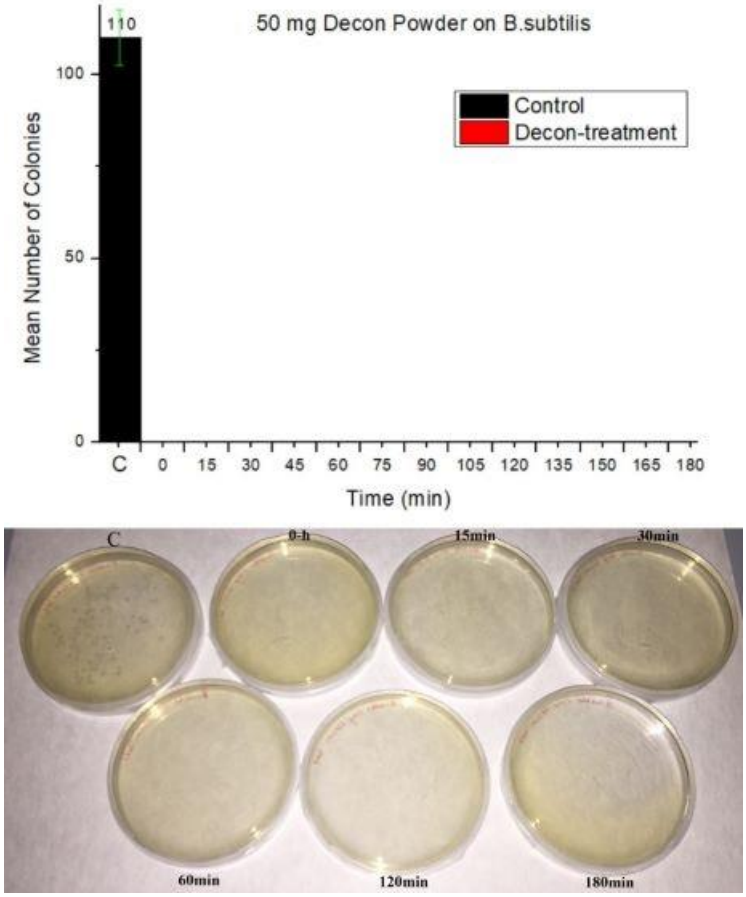

Figure 3.18 Mean number of CFU of 3 sets of B.subtilis treated with 50 mg decon powder.

Table 3.4 Mean CFU ± standart deviation, and percentage decrease (%) with respect to control groups for B.subtilis treated with decon powder.

B.subtilis	Control	0-h	15-min	30-min	60-min	120-min	180-min
750 mg	261±205,66	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)
500 mg	218 ± 17,62	0 (100%)	0 (100%)	0 (100%)	0,33±0,57 (99.86%)	0 (100%)	0 (100%)
250 mg	185 ±26,63	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)	8,3±14,43 (95.68%)
100 mg	273 ±34,59	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)
50 mg	110 ± 7,5	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)

As opposed to *E.coli,* we can state that even 50 mg decon powder had immediate effect for preventing the visible growth of vegetative *B.subtilis* cells (Table 3.4).

3.2.5 Antimicrobial Effect of Zeolite Powder on *B. subtilis* Spores

Sporulation of *Bacillus subtilis* normally occurs when nutrient levels are reduced or there are toxic substances in the environment in which these bacteria live (Setlow, 2005). Bacterial endospores are resistant to desiccation because in spore core, the water is displaced with Ca^{2+} - dipicolinic acid, and although not immune to all chemicals, they are more resistant to UV radiation (McKenney et al., 2013). In addition, spores cannot be neutralized by antibiotics (Louie et al., 2012). However, it was reported that even though treatment of such spores with peroxide or extreme heat would not suffice to destroy them, 10% bleach and autoclave sterilization would kill spores (McKenney et al., 2013). Determination of MIC by decon powder is therefore of special importance since, as explained, bacterial spores are very hard to neutralize owing to their complex structures (Figure 3.19).

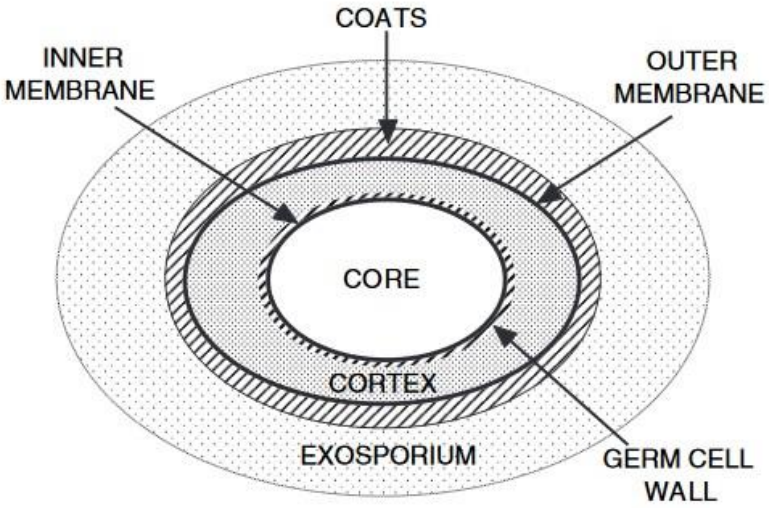

Figure 3.19 Different spore layers are depicted, size of some layers can show difference according to species (Taken from Setlow, 2005).

After sporulation was conducted as explained in Section 2.4, malachite green staining was performed in order to prove the existence of spores. *B.subtilis* cells in spore and vegetative states were detected by Floid Cell Imaging Station (Figures 3.20 and 3.21).

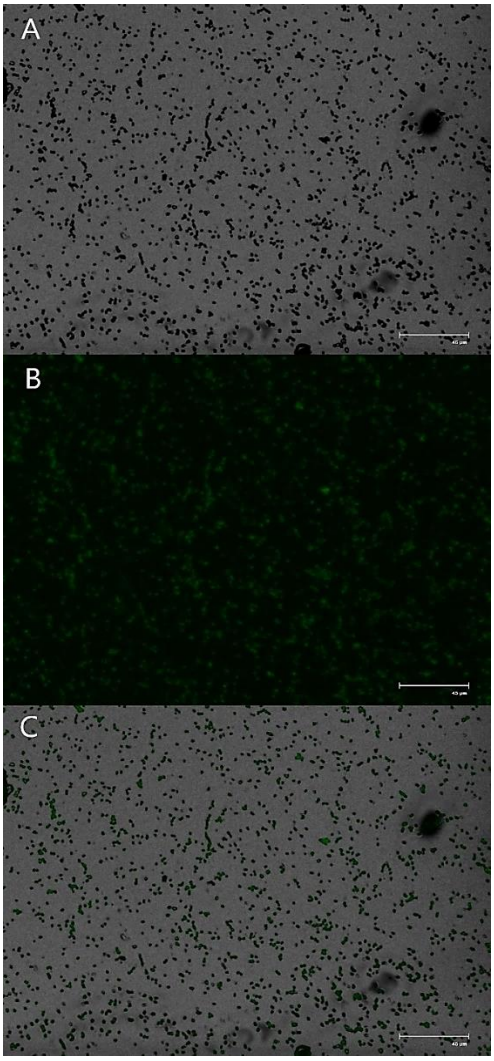

Figure 3.20 EVOS Floid Imaging Station (Thermo Fisher Scientific, USA) fluorescence micrographs of B.subtilis spores stained by malachite green solution with white (A) and green (B) channels and merge (C). Scale bars are 45μm.

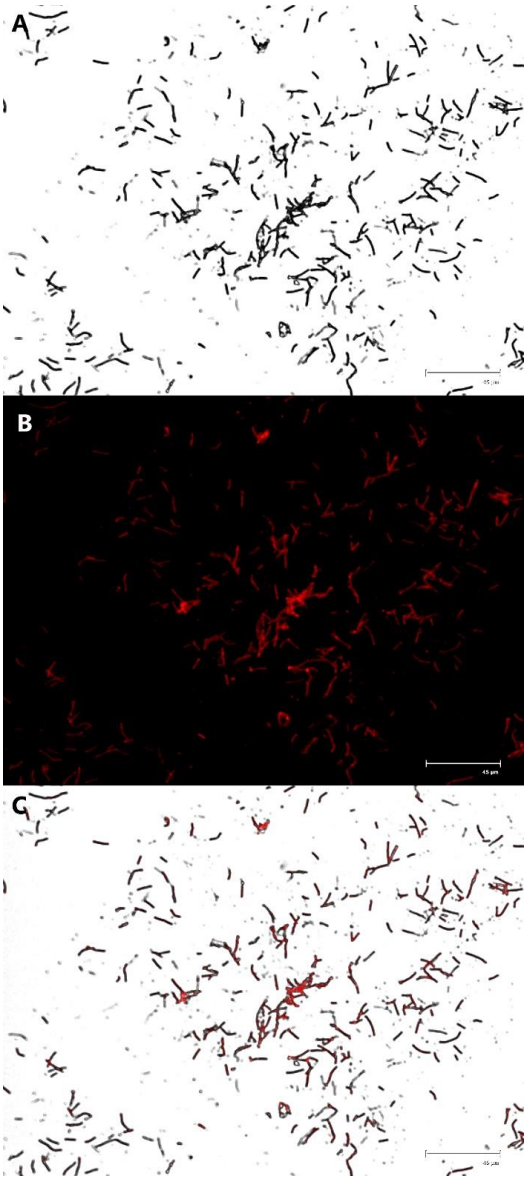

Figure 3.21 EVOS Floid Imaging Station (Thermo Fisher Scientific, USA) fluorescence micrographs of B.subtilis cells in vegetative state stained by safranin solution with white (A) and red (B) channels and merge (C). Scale bars are 45µm.

Since *B.subtilis* cells in vegetative state were more susceptible to the antibacterial action of decon powder compared to *E.coli*, we started the experiments with 500 mg decon powder (Figure 3.22). In 0-h plates, 90% reduction in colonies was observed, and throughout 180 minutes of powder treatment, we observed 99% reduction in number of colonies initiated by spores (Table 3.5).

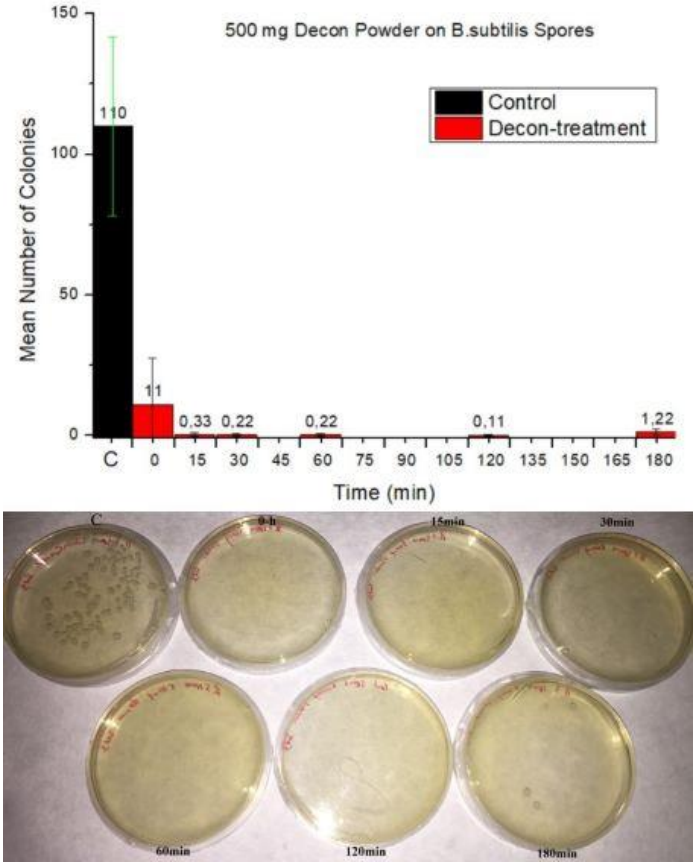

Figure 3.22 Mean number of CFU of 9 sets of B.subtilis spores treated with 500 mg decon powder.

Next, the dosage was reduced by half, and significant decrease in antibacterial action was observed (Figure 3.23). In all 3 sets, a spike in CFU for 120-min samples was seen. Most significant reduction (89.74%) was achieved only after 180 minutes of decon powder treatment (Table 3.5).

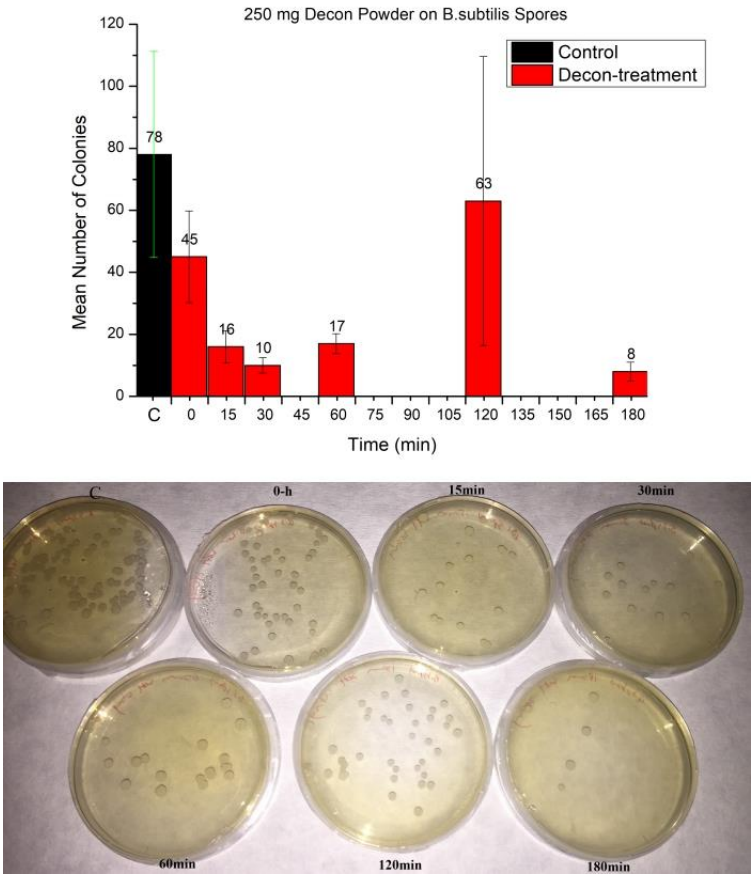

Figure 3.23 Mean number of CFU of 3 sets of B.subtilis spores treated with 250 mg decon powder.

We also wanted to observe whether the colony numbers would keep increasing if the dosage is decreased to 100 mg decon powder. As seen in Figure 3.24, compared to 250 mg decon powder, with 180-min plates, there was only 12.35% decrease in number of CFU (Table 3.5). Fluctuation in colony numbers throughout 0-h, 15-min, 30-min, 60-min, 120-min, and 180-min suggests that reliable antibacterial effect could not be achieved.

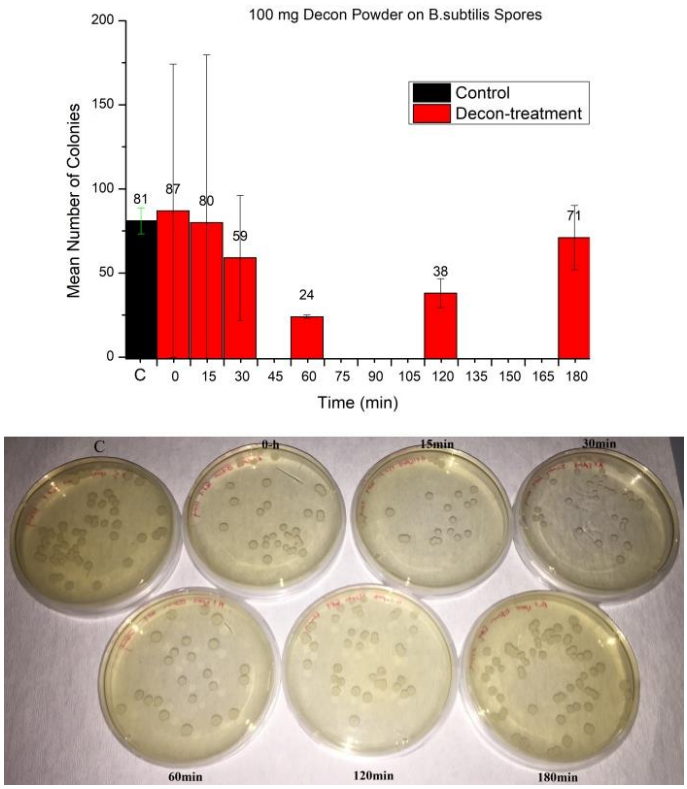

Figure 3.24 Mean number of CFU of 3 sets of B.subtilis spores treated with 100 mg decon powder.

Table 3.5 Mean CFU ± standart deviation, and percentage decrease (%) with respect to control groups for B.subtilis endospores treated with decon powder.

Endospores	Control	0-h	15-min	30-min	60-min	120-min	180-min
500 mg	110±31.86	11±16.55 (90%)	0.33±0.67 (99.73%)	0.22±0.42 (99.82%)	0,22±0.42 (99.82%)	0.11±0.31 (99.90%)	1.22±1.13 (99.10%)
250 mg	78 ± 33.2	45±14.80 (42.30%)	16 ± 5.2 (79.50%)	10 ± 2.52 (87.18%)	17 ± 3.21 (78.21%)	63 ± 46.6 (19.23%)	8 ± 3.05 (89.74%)
100 mg	81 ± 7.81	87±87.18 (no decrease)	80 ±99.60 (1.23%)	59 ±37.26 (27.16%)	24 ± 1 (70.37%)	38 ± 8.66 (53.10%)	71 ±19.14 (12.35%)

According to Table 3.5, it can be said that MIC_{99} value for spore neutralizing effect of decon powder is 500 mg. However, as observed, that effect could only be achieved with 15 minutes of direct contact between decon powder and spores. Throughout 180 minutes, there was no significant reduction in CFU amount.

3.2.6 Antimicrobial Effect of Zeolite Powder with Protamine on *B.subtilis* Spores

As performed with *E.coli* and *B.subtilis* cells in vegetative state, spores were also subjected to protamine. Two different mixtures were prepared and applied on spores. As seen in Figure 3.25, 200 mg decon powder mixed with 50 mg protamine prevented visible growth of spores even in 0-h plates. When 90 mg decon powder was mixed with 10 mg protamine, 99% reduction in colony numbers was observed (Figure 3.26).

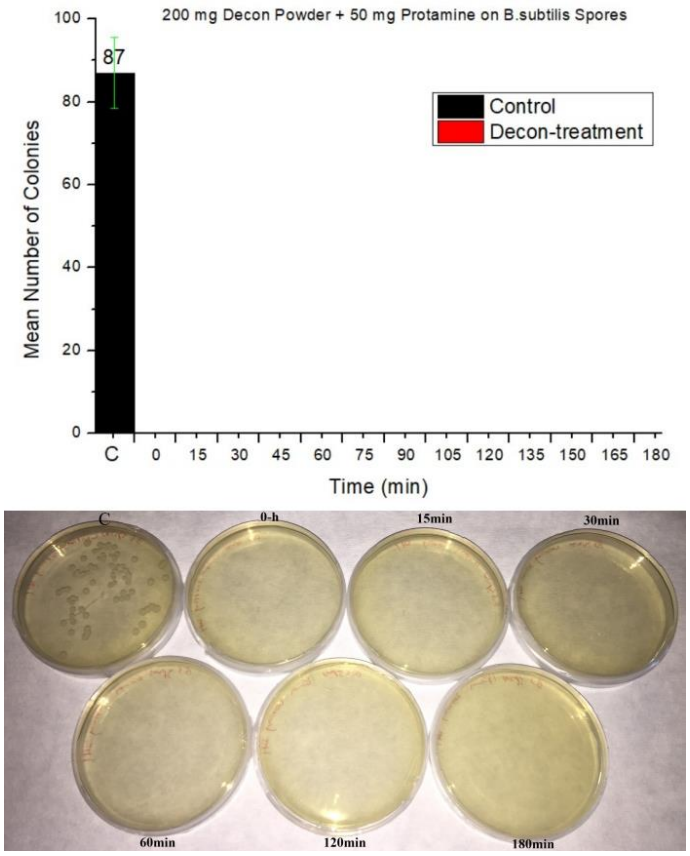

Figure 3.25 Mean number of CFU of 3 sets of B.subtilis spores treated with the mixture of 200 mg decon powder and 50 mg protamine.

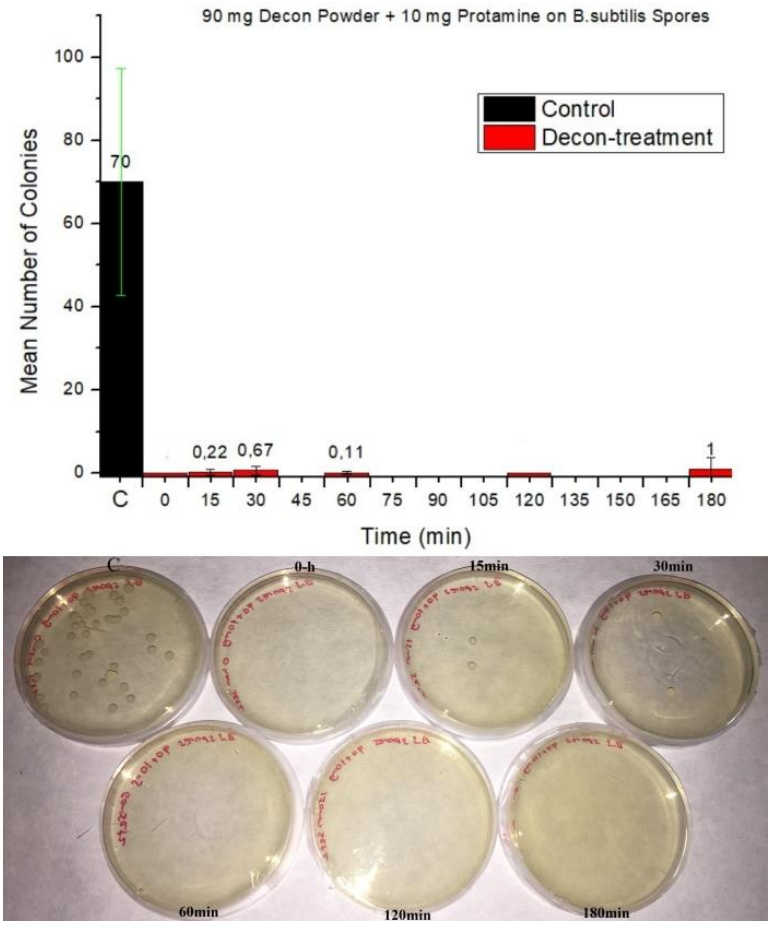

Figure 3.26 Mean number of CFU of 9 sets of B.subtilis spores treated with the mixture of 90 mg decon powder and 10 mg protamine.

According to Table 3.6, it can be said that MIC_{99} value for neutralizing the spores with mixture of the two compounds is the application of 90 mg decon powder and 10 mg protamine.

Table 3.6 Mean CFU ± standart deviation, and percentage decrease (%) with respect to control groups for B.subtilis endospores treated with decon powder and protamine together.

Endospores	Control	0-h	15-min	30-min	60-min	120-min	180-min
200 mg decon powder + 50 mg protamine	87 ± 8.50	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)
90 mg decon powder + 10 mg protamine	70 ± 27.3	0 (100%)	0.22±0.63 (99.71%)	0.67±1.05 (99.07%)	0.11±0.31 (99.86%)	0 (100%)	1 ± 2.83 (98.58%)

3.2.7 Antimicrobial Effect of Protamine on *B. subtilis* Spores

Lastly, we wanted to observe the antibacterial action of 10 mg protamine on spores in order to decide if there is synergistic interaction here as well like the one observed with *E.coli.* From Figure 3.27 and Table 3.7, it can be seen that 10 mg protamine prevented the visible colony development. So, the reduction in colony numbers from the mixture in Figure 3.26 could directly result from the action of protamine alone. We observed that in order to inhibit the activation and growth *B.subtilis* spores, 500 mg decon powder or 10 mg protamine are needed for at least 99% reduction in number of CFU.

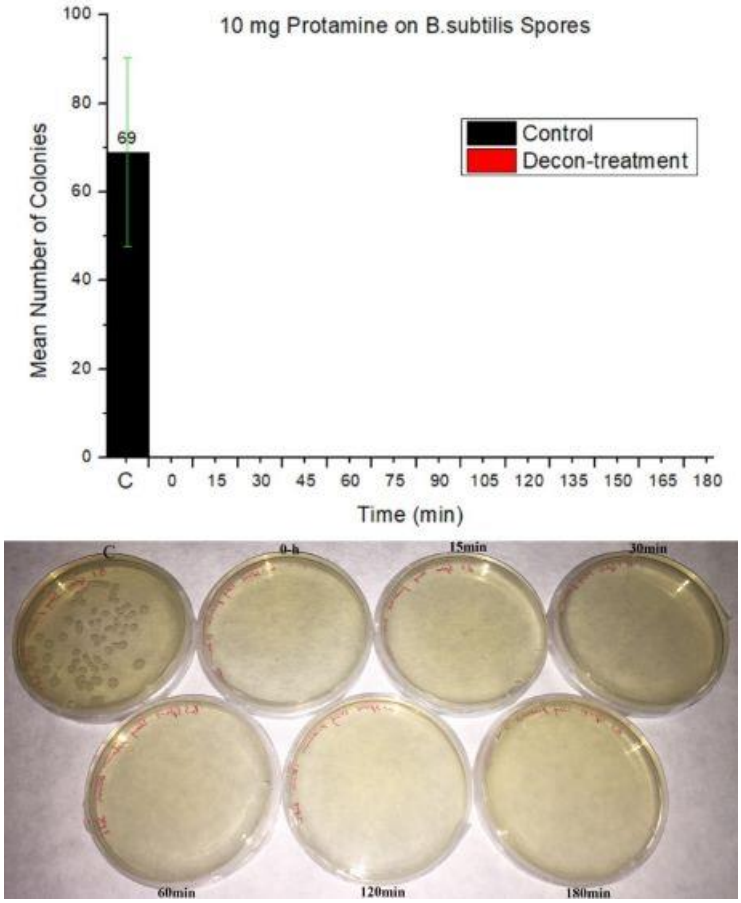

Figure 3.27 Mean number of CFU of 3 sets of B.subtilis spores treated with 10 mg protamine.

Table 3.7 Mean CFU ± standart deviation, and percentage decrease (%) with respect to control groups for B.subtilis endospores treated with protamine.

Endospores	Control	0-h	15-min	30-min	60-min	120-min	180-min
10 mg protamine	69±21.38	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)	0 (100%)

Gram positive and Gram negative bacteria differ in their cell wall structure. Gram positive bacteria have thick peptidoglycan layer in their cell walls, whereas Gram negative bacteria possess much thinner peptidoglycan layer covered by outer lipopolysaccharide (LPS) membrane. Thick peptidoglycan layer in Gram positive bacteria has been known to absorb antibiotics and antimicrobials, which makes it easier for such compounds to kill them, as observed in this study with *B.subtilis* (Breijyeh et al., 2020). Protamine is thought to exert antimicrobial effect through several mechanisms such as disruption in both cell membrane and cell wall by electrostatic interaction since protamine is positively charged as opposed to bacterial cell membrane and cell wall, causing leakage of cellular materials by inducing small pores (Kim et al., 2015). AgNPs in decon powder is known to kill bacteria by altering membrane structure such as permeability and cellular transport, however perhaps the more potent bactericidal effect is that AgNPs could interact with ribosomes, which results in denaturation of them and thus, translation is inhibited (Dakal et al, 2016). Another mechanism of antibacterial action reported was, once inside the cells, silver ions can cause condensation of DNA, thus inhibiting replication (Dakal et al, 2016). Zinc oxide and titanium dioxide were thought to cause the release of free radicals by hydrogen peroxide production in bacteria (Xie at al., 2011 ; Ahmed et al., 2020). So, combining the active ingredients in decon powder and protamine, it can be proposed

that once protamine causes small perforations in cell walls and membranes of bacteria, through these pores, AgNPs, along with ZnO and TiO_2 , enter the cytoplasm of bacteria, and thereby kill them.

This explains why *E.coli* is more resistant to low doses of decon powder compared to *B.subtilis*. Outer LPS is responsible for this resistance. First of all, this outer layer would not stick to (absorb) antimicrobials, and second, these compounds cannot easily pass through LPS. This is the reason for synergistic interaction between protamine and decon powder. Protamine should cause perforations on cell wall so that antimicrobial compounds in zeolite powder could get a pass into the cells.

Exosporium and spore coats are the major barriers that renders most antibiotics useless to kill bacterial spores. *B.subtilis* spores, however, do not possess exosporium layer (Stewart, 2015). Spore coat, which is a proteinaceous layer, was reported to play a role in germination of *B.subtilis* cells, so the damage to this part might prevent activation of spores (McKenney et al., 2013). Surrounded by this coat, cortex layer , is actually made of proteoglycan (Figure 3.19). Use of protamine and decon powder together might have damaged the spore coat in lower doses by synergistic interaction, preventing reactivation of spores when conditions became favorable.

3.3 Investigating Blood Coagulation Effect of Decon Powder

Using whole blood samples, different concentrations of sodium alginate, gelatin, protamine and chitosan solutions were prepared and tested in order to compare coagulative properties, or more accurately expressed as induction of erythrocyte aggregation, with decon powder. Preliminary studies performed with erythrocyte suspension allowed concentrations of these solutions to be adjusted.

Since decon powder investigated here is intended to be used on skin, we wanted to find out if it possesses coagulative properties as well. The difference is that only decon powder was in solid form, other chemicals were dissolved in distilled water. Whole blood sample used was treated with EDTA to prevent coagulation in tubes.

From Figure 3.28, it can be observed that 1.5% chitosan impregnated gauzes released the minimum concentration of hemoglobin. 3% chitosan impregnated gauzes were not as effective.

Compared to standard gauze, alginate, gelatin and protamine impregnated gauzes showed coagulatory properties to withhold the blood as well.

Decon powder could also induce blood coagulation. In fact, OD values are more consistent compared to other gauzes throughout 3 experimental groups. The results indicate that decon powder can be used to absorb and withhold the blood in vitro.

In order to confirm that there is a significant difference between standard gauze and decon powder (distributed on gauze) with respect to their abilities to withhold 100 µL blood samples, two-sample t-test was performed. It is observed that there is a statistically meaningful difference between two groups (Figure 3.29).

Lastly, to see if there would be significant difference in hemoglobin concentration released, solutions of alginate, protamine, gelatin and chitosan were mixed in equal volume in which the gauze was soaked. As seen in Figure 3.28C, no meaningful difference compared to chitosan impregnated gauze was observed.

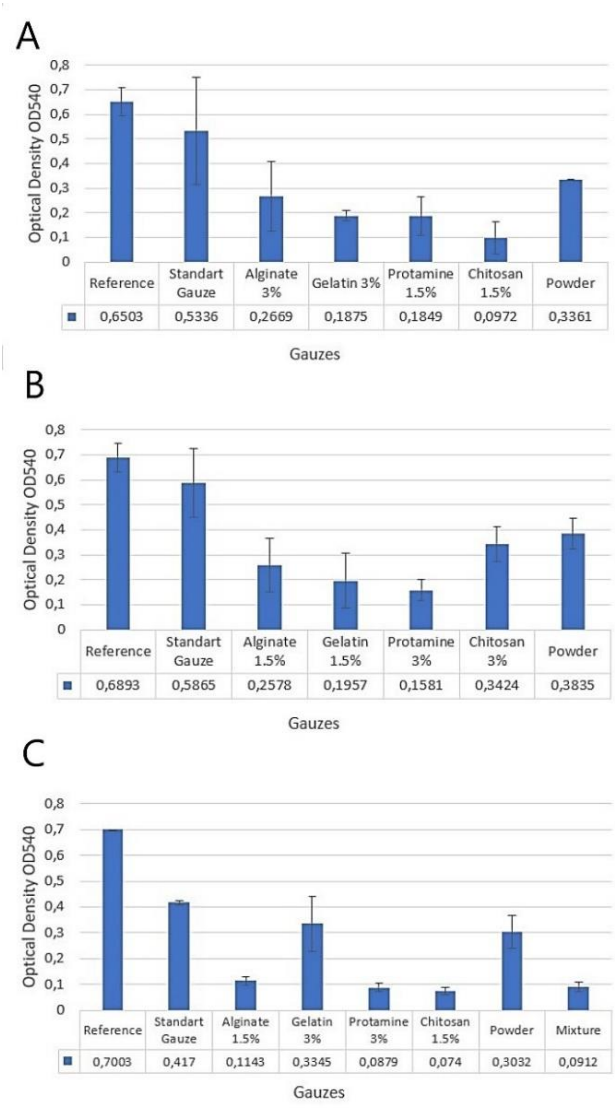

Figure 3.28 (A,B,C) Spectrophotometric readings of 3 different experimental groups (in duplicates). 500 mg decon powder was distributed on 1x1 cm gauze in each.

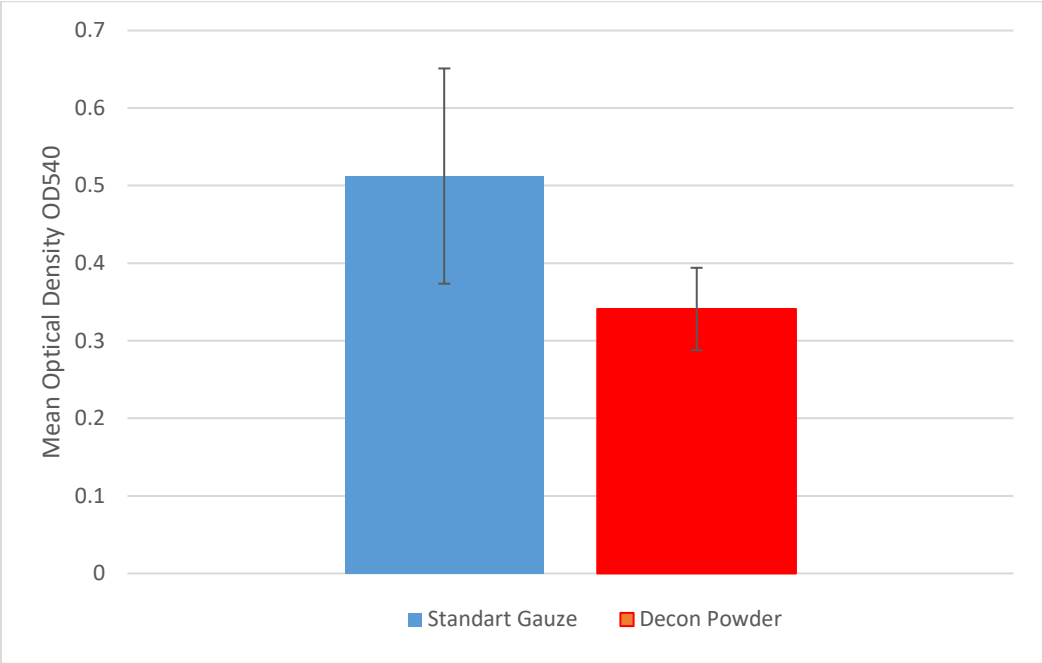

Figure 3.29 Hemoglobin concentration released by standard gauze and decon powder treated gauzes. Amount of hemoglobin released by standart gauze (M = 0.5123, SD = 0.1387, n =6) was hypothesized to be greater than that of decon powder treated gauze (M = 0.3409, SD = 0.0531, n=6). This difference was significant, t (8) = 2.3060, p= .027 (1 tail). p < 0.05

CHAPTER 4

CONCLUSION

Decontamination in context of CBRN Defense is an absolute necessity to stop limiting the spread of contaminants that would keep inflicting additional damage to people if not dealt with. Decontamination can be carried out by neutralizing the toxic compounds that are of biological and chemical origin, or by simply removing from specific places and containing them safely in case of radiological agents. In this thesis, biological aspect of decontamination was focused on. Many of the commercial products that exist for such incidents are not suitable for use on human body, so there is a need for development of such materials. We aimed test the antibacterial action of such material made of zeolite powder. The advantage of using a decontaminant in solid (powder) form is that the amount of such product to be used can be adjusted easily. Its application and transportation would not be troublesome either compared to decontaminants in liquid form, which have a relatively short shelf-life.

We used two different species of bacteria, one of them represented the Gram-negative pathogens and the other, Gram-positives. In addition, we also tested the powder on *B. subtilis* spores as well, as these dormant structures are very hard to destroy compared to vegetative cells.

In order to observe if antibacterial effect could be increased by synergistic interaction, we also used protamine sulfate alone and in mixture with decon powder.

We also took into account of time variable, meaning in every set, 6 different timeline was used up to 180 minutes. Increasing the contact time between decon powder and

bacterial samples only showed decrease in CFU in lower doses for both decon powder and protamine.

For *E.coli*, Minimum Inhibitory Concentration was observed to be 100 mg given 60 minutes, and 500 mg given 15 minutes.

Mixing protamine with decon powder increased the antibacterial action considerably. Lowest dose of mixture tried was 90 mg decon powder and 10 mg protamine. No colony development was observed in any of the plates. Synergistic interaction between protamine and decon powder formulation were thus apparent.

Protamine alone was also tested for comparison. Although with 50 mg protamine, immediate antibacterial effect was observed within minutes, reducing the dose to 10 mg required 60 minutes of contact time for 100% reduction in visible colony development.

B.subtilis, on the other hand, showed much greater susceptibility to decon powder. Even only 50 mg powder showed immediate antibacterial action with 100% reduction in CFU number.

Decon powder formulation could prevent the activation and significantly reduce the visible growth of endospores with 500 mg dose and 100% reduction in growth was observed at 200 mg decon powder mixture with 50 mg protamine.

Using 200 mg decon powder mixed with 50 mg protamine on spores showed immediate antimicrobial action with 100% reduction in colony development. However, when the dose was adjusted to 90 mg decon powder with 10 mg protamine, several colony developments were seen in some of the plates. Having said that, the reduction in colony numbers reached 99.86%.

By its nature, CBRN incidents can be unpredictable and violent. Decon powder is intended to be used on skin, so if skin integrity is compromised, along with antibacterial action, we wanted to see if it can help with reducing superficial bleedings as well. To test that, we designed an experimental setting that measured powder's ability to induce clotting by erythrocyte aggregation. Alginate, gelatin, protamine and chitosan solutions were used as well for comparison. We found a statistically significant difference that decon powder treated gauzes would release less hemoglobin to surrounding with respect to standard gauzes.

During decontamination efforts in CBRN incidents, 'golden minute rule' is very important. This rule states that decontamination of victims should be performed as quickly as possible, preferably within a minute of contamination. Owing to its zeolite structure, decon powder has the ability to quickly and thoroughly absorb liquid contaminations.

Although not shown in this study, decon powder investigated here was designed to be used on human skin. One advantage of utilizing decon powder formulation therefore is that it is a cost-effective alternative of many of the commercially available liquid decontaminants. Another advantage is its long shelf life, and zeolite powder formulation could also withstand high temperatures without losing effectiveness. However, because it is insoluble in liquids, testing the antimicrobial effect of such materials require specially designed experimental procedures.

Considering the results, we can state that decontamination powder investigated here possesses both antibacterial and coagulation inducing effects in vitro. This study also offers a novel procedure for testing solid, non-dissolvable antimicrobials on bacterial and spore samples.

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