Resuscitation of Bacterial Biofilm by Sunlight: Effects on Different Enteropathogenic Bacteria

By:

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of

Master of Science in Biotechnology

Department of Mathematics and Natural Sciences

BRAC University

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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing Maters of Science degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

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Approval

The thesis titled "Resuscitation of Bacterial Biofilm by Sunlight: Effects on Vibrio cholerae, Salmonella typhi and Shiga Toxin Producing Escherichia coli" submitted by

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has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Masters in Biotechnology on [Date-of-Defense].

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Ethics Statement

This material is an original work, which has not been previously published elsewhere. It is my own research and analysis in a truthful and complete manner. The paper properly credits all the sources used (correct citation).

Abstract

It is an established fact that most bacteria go through motile and sessile form during its lifetime. This transition has significant roles in seasonal outbreaks of certain bacterial diseases and bacterial pathogenesis. There are many factors that influence the making and breaking of biofilms. Breakage of biofilms gives rise to more planktonic bacteria in the waters. This resuscitation makes the bacteria more capable to infect. In this study we investigated if sunlight has any effect on breaking biofilms of seasonal disease causing bacteria. Biofilms of several strains of bacteria that significantly cause disease during March to July in the tropical zones were subjected to sunlight exposure in different manners. The results indicated that exposure in sunlight significantly break down the biofilms of all the bacteria. This could be one of the reasons for the seasonal epidemics. Because breakage of biofilms give rise to more planktonic bacteria in the waters. This resuscitation makes the bacteria more capable to infect. However, to reach any conclusion, round the year study including more samples is required.

This work is dedicated to

My Dear Family

Acknowledgement

First of all, I wish to declare my humble gratitude to Almighty Allah, who has bestowed the gift of outmost mercy, and given me the strength, patience and understanding required to complete this work amid this Covid- 19 situation.

I convey my special thanks to Professor A. F. M. Yusuf Haider, PhD, Chairman, Department of Mathematics and Natural Sciences, BRAC University, who always has tremendous support for every student. I express my gratitude to my supervisor, Iftekhar Bin Naser, PhD, Assistant Professor, Department of Mathematics and Natural Sciences, BRAC University, for his encouragement and continuous guidelines for this research. I am indebted to all the extraordinary people of the Life Science Laboratories, BRAC University. Without their all-time support this would not be complete. I would like to specially recognize Asma Afzal, Lab officer, Life Science Laboratories, BRAC University and Akash Ahmed, Lecturer, Department of Mathematics and Natural Sciences, BRAC University. I would like to express my deepest respect to Mahboob Hossain, PhD, Professor, Department of Mathematics and Natural Sciences, BRAC University for always being a great inspirer.

I sincerely thank my beautiful family who trusted in me and is always there for me. This journey would be incomplete without some dear friends. Saria Farheen, Fahim Sarwar and Nowrin Hossain were my greatest supports while I did this work. I am grateful to all of these people.

Sincerely,

Afia Nowshen,

Department of Mathematics and Natural Sciences, BRAC University

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List of Acronyms

STEC- Shiga Toxin Producing *E. coli*

EPS- Exo-Polysaccharide

VPS- *Vibro* Polysaccharide

CVEC- Conditionally Viable Environmental Cell

VBNC- Viable But Not Culturable

OD- Optical Density

ELISA- Enzyme Linked Immunosorbant Assay

EIA- Enzyme Immunoassay

RIA- Radial Immunoassay

ELISPOT- Enzyme Linked Immuno Spot Assay

HUS- Hemolytic Urine Syndrome

CTX- Cholera Toxin

ER- Endoplasmic Reticulum

AFR6- ADP ribosylation factor 6

c-AMP- Cyclic Adinosine Monophosphate

PKA- Phospho Kinase

CFTR- Cystic Fibrosis Trans membrane Receptor

LPS- Lipopolysaccharide

STX- Shiga Toxin

PP- Peyer's Patch

List of Acronyms

FERG- Foodborne Disease Burden Epidemiology Reference Group

AFR- Africa (WHO Classification)

AMR- America (WHO Classification)

EMR- Middle Eastern Region (WHO Classification)

EUR- Europe (WHO Classification)

SEAR- South-East Asia Region (WHO Classification)

WPR- Western Pacific Region (WHO Classification)

1. Introduction:

1.1 Background:

Biofilm formation is a common feature in many pathogenic bacteria. They enter this state as a survival mechanism. The complex microenvironment inside a biofilm allows the cells to survive in stress. There are many situations that encourage bacteria to become biofilm such as,

- Adverse environmental conditions,
- Lack of nutrients
- Phage predation (Faruque, 2005, Naser, 2017) etc.

However, they come back to planktonic state. When resuscitation occurs, bacteria become free from the biofilm matrix and become active. If pathogenic, they can cause disease if they get access to a host. This transition is significantly important in microbes. It helps to maintain their life cycle as well as pathogenesis. There are several factors responsible for this resuscitation. Some are known, and some are still to be known. Some known factors are,

- Removal of environmental stress
- Quorum sensing (Hoque, 2016)
- Auto- inducers (Hoque, 2016) etc.

From studies done before, we got the idea that sunlight may be a cause of seasonal resuscitation of bacteria. That is why diseases like cholera, typhoid and diarrhea are predominant during March to July in tropical regions. At this time the sunlight is very strong and stays for a long time. In this study we aim to put this theory to test.

1.2 Aims of the study:

The aim of this study is to investigate if sunlight works as a factor for breaking the biofilms and releasing the bacteria in planktonic form. If so, this could be one of the reasons behind seasonal outbreaks of diseases like cholera, typhoid and bloody diarrhea (hemorrhagic colitis) caused by STEC.

2. Literature Review

2.1 Biofilms:

Biofilm is a community of microbes that arrange themselves in a microenvironment that is self-sustaining. Bacteria attach to one another by different attachment factors and ultimately arrange themselves in a matrix. This matrix serves as the vehicle of the complex networking of nutrients, metabolites, genes etc. that goes on inside a biofilm.

Formation of biofilms is a universal attribute for most bacteria. *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis,* and *Staphylococcus aureus* are considered as model organisms to study biofilms (Lopaz 2010) Depending on the type of predominant bacteria in a biofilm, its attributes can differ.

Biofilms help helps them to survive harsh environment, lack of nutrition, physical or chemical stress, host immune system, antibiotics (Mosharraf, 2020) and even predators (Naser,2017). Biofilms can be made up of one or more than one type of bacteria. They can be formed on both living and non-living surfaces. They can be found anywhere like lake water, raw food, sewage lines, kitchen sinks, animal tooth, laboratory tool etc. Commonly biofilms are referred as slime. However, inside slime develops a unique and complex system that is stable and have significant role in microbes' survival and pathogenesis.

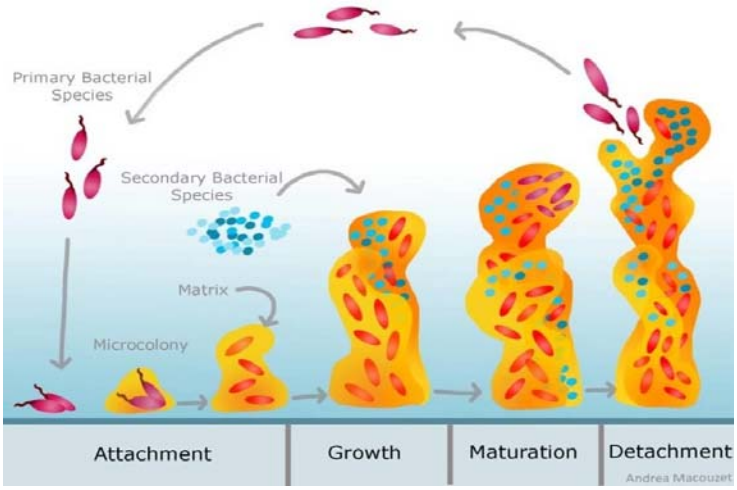


Image 2.1: Simplified Diagram of a Biofilm (Jacques, 2010)

Biofilms start to manifest itself when bacteria in a certain environment start to communicate and attached to each other. This attachment grows with time and gives rise to cell clusters. These cell clusters are then encapsulated into the matrix of the biofilms.

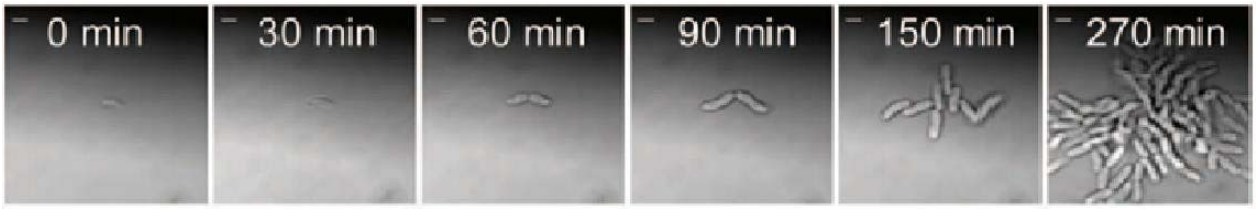


Image 2.2 Vibrio cell cluster formation (Berk, 2012)

Inside a biofilm, bacteria are organized in a self-produced unique architecture called the exo-polysaccharide (EPS) (Lopaz 2010). EPS is lattices like formations that gives biofilm a defensive advantage and allow the transport of supplements, enzymes, metabolites, and transfer of wastes inside and outside the biofilm matrix. (Mosharraf, 2020). EPS is addressed in many different ways depending on the type of biofilms. In case of *vibrio* biofilms this is known as VPS (Vibrio polysaccharide) (Berk, 2012) and they start to form as fast as 15 minutes of contact.

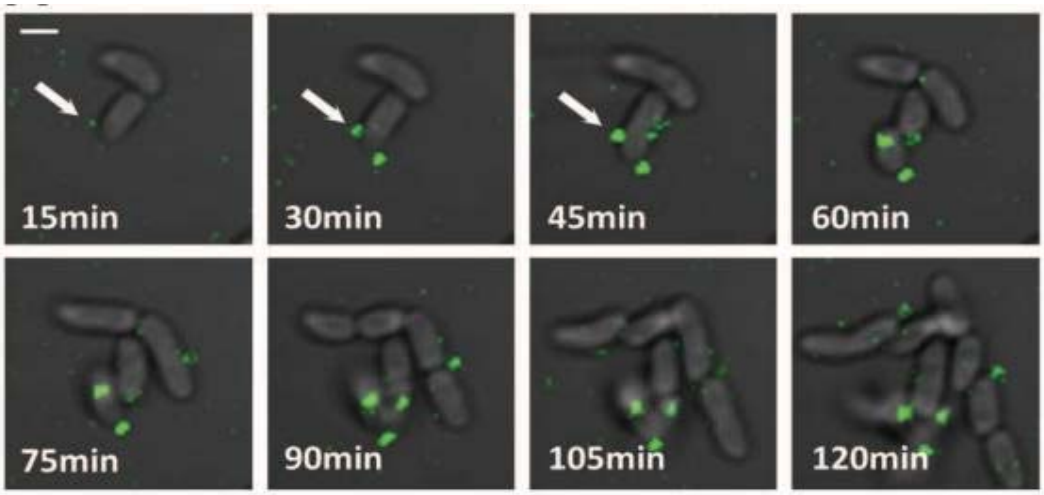


Image 2.3: Time lapse image of VPS (Vibrio polysaccharide) Secretion and cell attachment (Berk, 2012)

Other than this EPS, biofilm also contains different kinds of proteins, nucleic acids, peptidoglycan, lipids, phospholipids, and other cell components (Mosharraf, 2020). Together they give a biofilm stability.

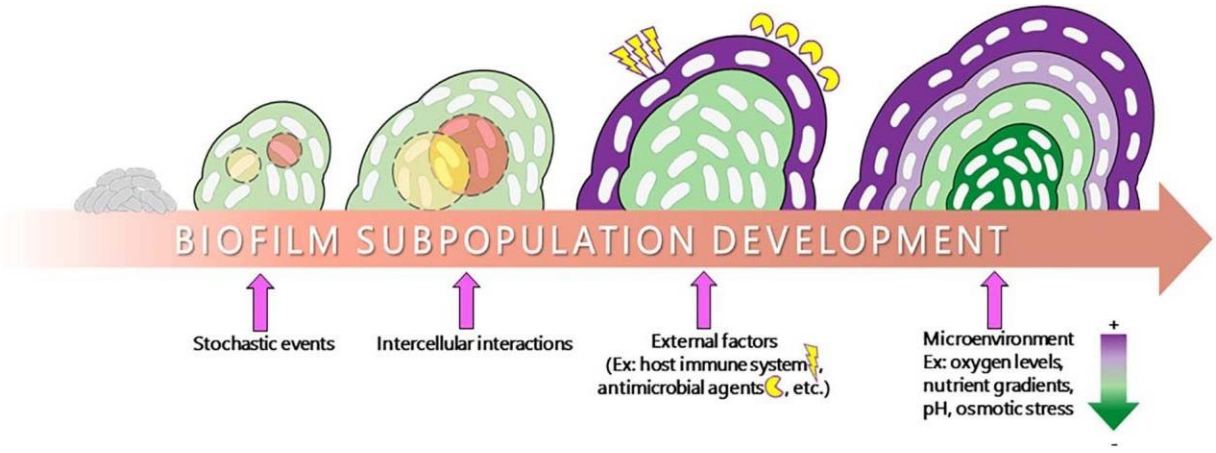


Image 2.4 Actions that take place inside a biofilm (Krishnama, 2019)

At one point of living inside a biofilm, bacteria can detach it and again come back to planktonic form. This depends on many internal or external factors. In this study it will be investigated if sunlight is one of them.

2.2 Pathogenic Significances of Biofilms

According to the National Institutes of Health, 80% of all infections in humans are related to biofilms. As biofilms have the capacity to evade antibiotics, stress even the immune system, biofilm-related infections are hard to cure with existing treatments (Mosharraf, 2020). Many bacteria that cause severe illness in human have the capacity to form biofilms. In fact, some of them have to go through transition between sessile and motile to actually cause a severe disease.

A good example for this is *Vibrio cholerae*. Motility allows this bacterium to reach and attach to the target site of infection. And biofilm form gives it the necessary resistance against the host. For infection the pathogen has to accomplish intestinal colonization, followed by dissemination and excretion inside the human body (Silva, 2016). That is possible when this kind of transition occur. In this way the infection spreads throughout the human intestine. When attached to target

cells, the pathogen inserts CT Toxin in the intestinal cells and cause damage. When excreted from patients, stools contain a mixture of slime, cluster and single cells of cholera (Silva, 2016).

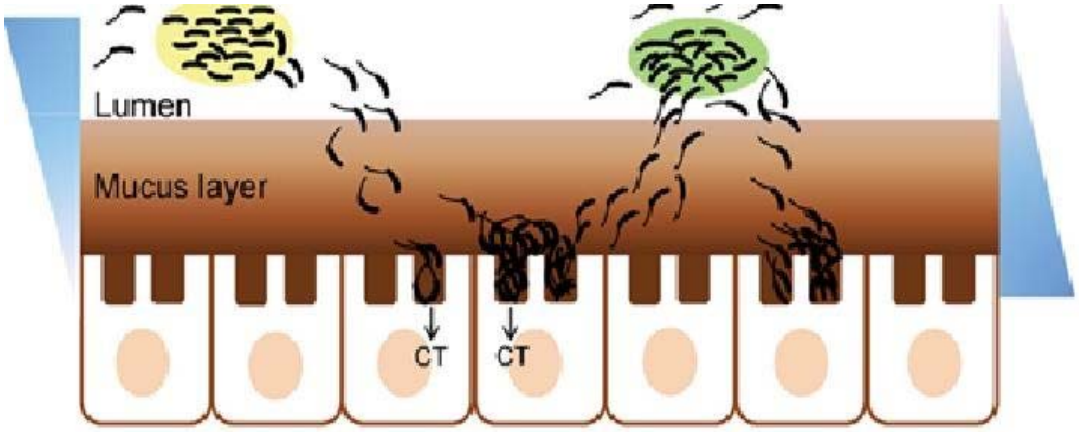


Image 2.5: Transitions of vibrio cholera between sessile and motile form (Silva, 2016).

Both motility and biofilm form give this bacteria unique fitness to cause the disease. For instance, when motile, *vibrio* can swim towards the mucus and attach and penetrate that. It can also help the vibrio to stick to the cell surface and release CT Toxin (Hoque, 2016). While sessile form has enhanced infectivity, resistance against bile and antimicrobial agents (Silva, 2016). It can also withstand mechanical cleansing. Thus, both scenarios are necessary for an effective infection

2.3.1 Vibrio cholerae

V. cholerae is a pathogen that causes diarrhoeal diseases in human that can be fatal. There are many serogroups of V. cholerae. Two of them known as O1 and O139 are known to cause severe disease (Faruque, 2005). However, *V. cholerae* species is largely comprised of non-toxigenic strains. Environmental strains display a high degree of genetic variability which suggests that *V. cholerae* is significantly environmental stress resistant and has significant interactions with other components of the aquatic environment like fish, algae, crustaceans etc. (Baker, 2018), (Carla, 2013). In the aquatic environment *V. cholerae* remains as aggregates of dormant cells called

CVEC (Conditionally Viable Environmental Cells), or as biofilms where most of the cells are at VBNC (Viable But Non Culturable) state (Faruque, 2006) (Hoque, 2016).

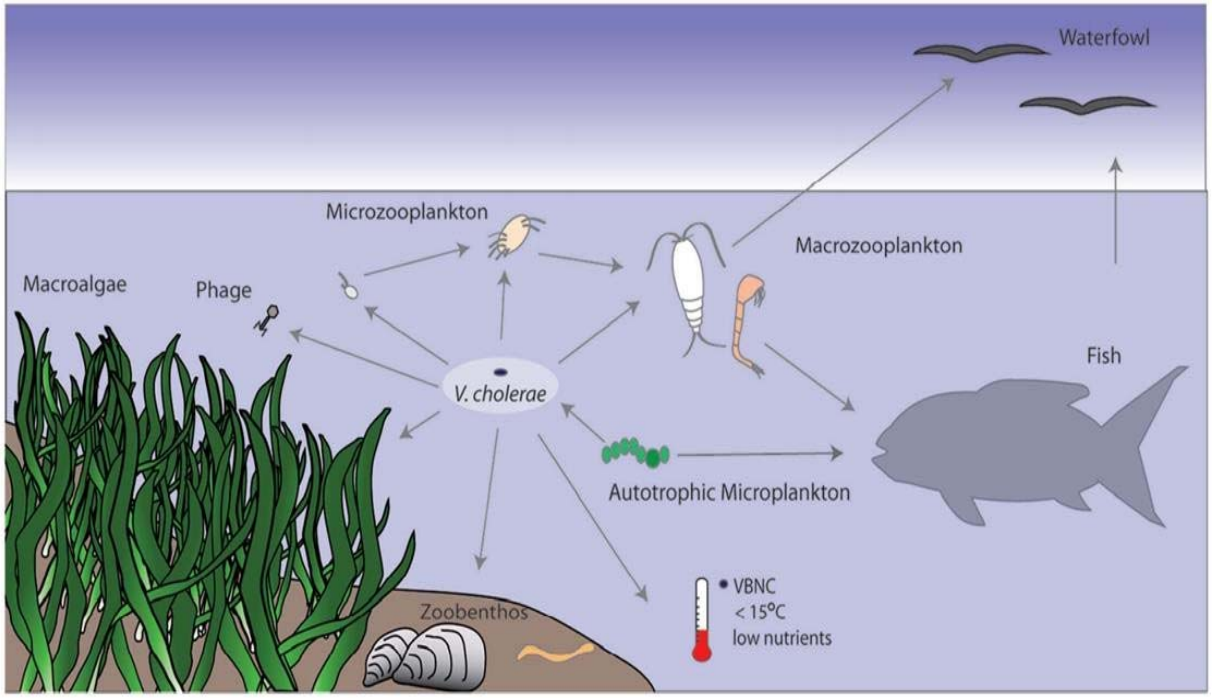


Image 2.6: Interactions of *V. cholerae* in the aquatic environment (Carla, 2013).

V. cholerae O139 was first identified in Bangladesh in 1992 (WHO, 2021). Though it is considered a common flora of water in Bangladesh, it can become dangerous during some seasons. This pathogen is important not only in Bangladesh, but also for many other countries. North and Middle America, South America, Europe, Middle East and South Africa are places where *V. cholerae* have cause infections (Carla, 2013). A map was constructed to show the distribution of *Vibrio* across the globe that comprised of many studies across the world:

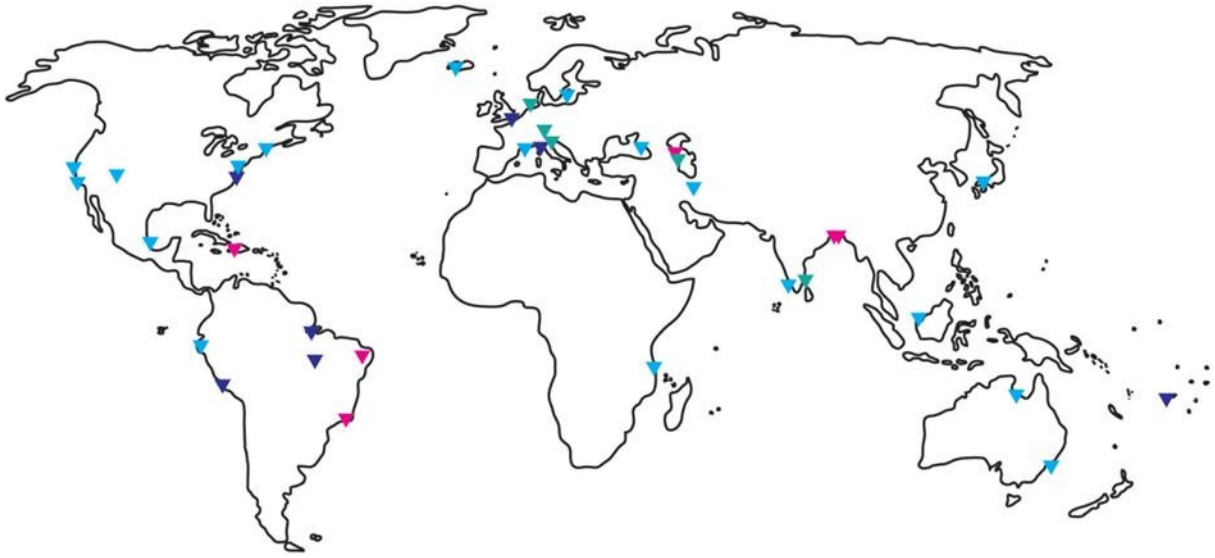


Image 2.7: Global Distribution of *V. cholerae*. Triangles indicate where *V. cholerae* was detected by molecular and/or culture-based methods. Red indicates O1/O139 detection, light blue non-O1/non-O139 detection, and dark blue did not specify (Carla, 2013)

V. cholerae is an important pathogen for the human history. In the 1960s *V. cholerae* started from the Bay of Bengal and spread in independent but overlapping waves (Baker, 2018). Different strains of this pathogen had significant emergence episodes in South America, the United States, Russia, France, Italy and Spain at different times (Baker, 2018). It is still widely studied across the world.

2.3.2 Cholera

Researchers have estimated that each year there are 1.3 to 4.0 million cases of cholera, and 21 000 to 143 000 deaths worldwide (WHO, 2021). Dehydration due to diarrhea is a major cause of deaths.

Most of the infections caused by cholera will come no symptoms or mild symptoms like watery diarrhea. It takes between 12 hours and 5 days after ingesting contaminated food or water to show symptoms. Infection clears out within 10 days usually (WHO, 2021). Most infections can

be successfully treated with oral rehydration solution. But antibiotics can be necessary if the illness is severe.

Cholera pathogenesis follows a certain pathway. The pathogen enters the human host by contaminated water. After reaching and harboring the target organ (small intestine), *Vibrio cholerae* begins expressing virulence factors, like cholera toxin.

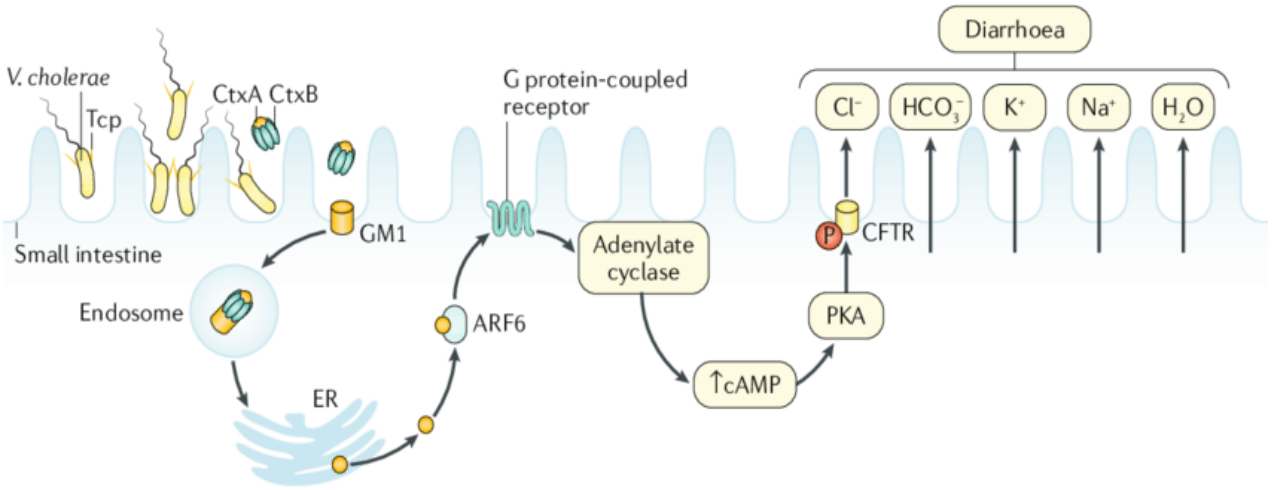


Image 2.8: Cholera Pathogenesis (Baker, 2018)

Cholera toxin is composed of two subunits, CtxA and CtxB, and binds to the ganglioside GM1 on the plasma membrane of cell via the CtxB pentameric subunit. GM1 bound cholera toxin is then engulfed by the cell, inside the cell the complex is transported to the endoplasmic reticulum (ER). There the CtxA and CtxB subunits dissociate from each other. CtxA is an enzymatic subunit. When released from the ER into the cytoplasm and its allosteric activation by ADP ribosylation factor 6 (ARF6) is activated (Baker, 2018).

The ARF6- CtxA complex activates Adenylyl cyclase by catalysing a G protein-coupled receptor. This increases the levels of cAMP in the cell and lead to protein phosphorylation (P) of the cystic fibrosis trans-membrane receptor (CFTR). This in turn makes an efflux of ions and water into small intestinal lumen which results in watery diarrhea (Baker, 2018).

2.4.1 STEC

Shiga toxin-producing *E. coli* (STEC), also referred to as Vero cytotoxin-producing *E. coli* (VTEC) or Enter Hemorrhagic E. coli (EHEC) is the most common type of pathogenic *E. coli*. (CDC, 2014). This bacteria cause foodborne outbreaks in tropical regions of the world and has significant implications on public health (Nastasijevic, 2020). STEC produces Shiga-toxins that are similar to that of *Shigella*. It can grow in temperatures ranging from 7 °C to 50 °C, with an optimum temperature of 37 °C (WHO, 2021)

STEC is distributed in many regions of the world. FERG, Foodborne Disease Burden Epidemiology Reference Group collected STEC related data from 21 countries and regions with and develop many analysis on foodborne illness by region (WHO, 2018). According to them, the distribution of STEC in the world looks like following:

Image 2.9: Disease burden (DALYs) of STEC by sub-region, 2010 (Kirk, 2015)

According to FERG, STEC is known to take different routes of transmission. They calculated the data and found out that, Across all sub-regions, about half of the STEC disease burden was estimated to be foodborne, with 1.2 million new cases resulting in 128 foodborne deaths (WHO, 2018).

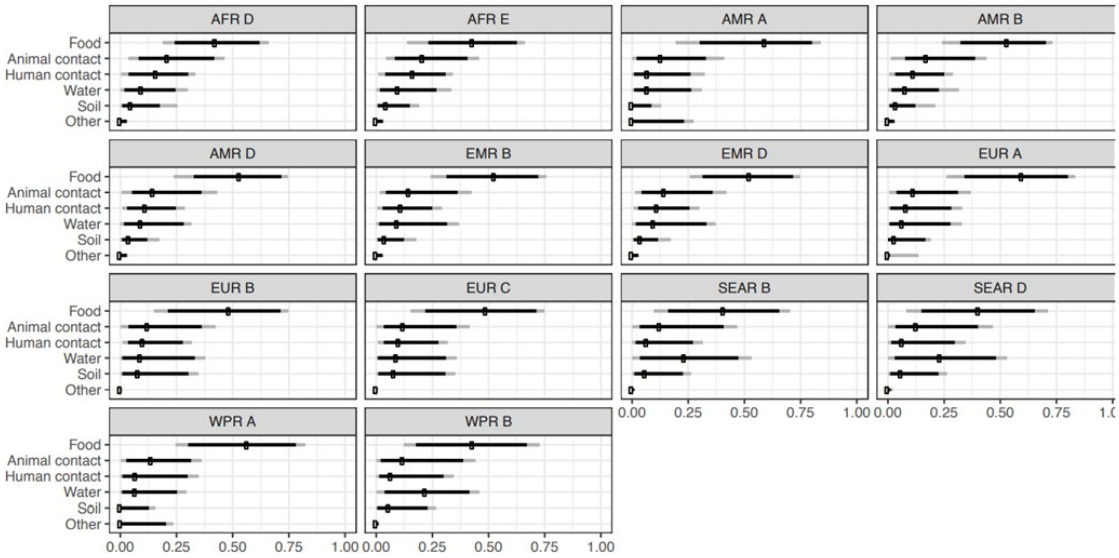


Image 2.10: Different routes of transmission for STEC infection by WHO classified sub-region in the world (Hald, 2016)(WHO, 2018)

STEC is still a big concern in the world and often considered a neglected tropical disease (NTD). The most effective method of STEC control is cooking food and boiling water over 70 °C Temperature.

2.4.2 Diseases caused by STEC

STEC can cause different kinds of complications in human body.

Symptoms vary from simple abdominal cramps to HUS (haemolytic uraemic syndrome) that is characterized by acute renal failure, haemolytic anaemia and thrombocytopenia (low blood platelets) (WHO, 2021). Fever, diarrhea and vomiting are common symptoms of STEC infection. This organism can take 3 to 8 days for incubation and most patients recover within 10 days (WHO, 2021) Very young kinds and elderly are the highest risk group for this disease. HUS develops in these patients mainly and may become life threatening.

STEC infection has a case-fatality rate ranging from 3 to 5% with HUS is the most common cause of acute renal failure in young children. Neurological complications (such as seizure, stroke and coma) can develop in 25% of HUS patients (WHO, 2021)

STEC pathogenesis follows a certain pathway that ultimately results in profound cell and organ damage by activation of immune cells if not treated at an early stage of infection.

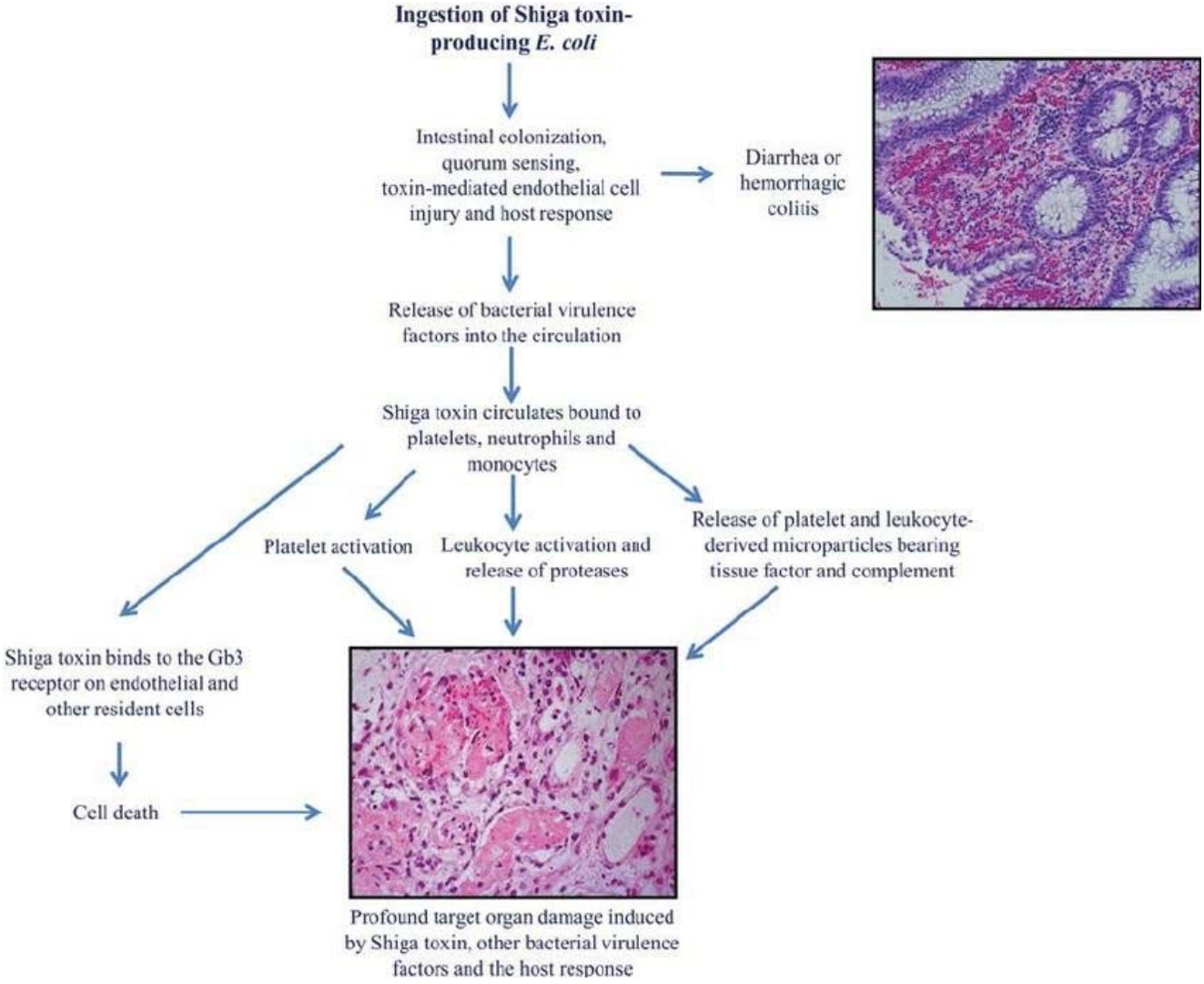


Image 2.11: STEC Pathogenesis (Karpman, 2012)

Terminal ileum and follicle-associated epithelium of Peyer's patches is the primary infection site of STEC. After ingestion, they reach the target sit and start to colonize. This event is further encouraged by quorum sensing. When bacterial colony reaches an optimal population, they release virulence factors such as shiga toxin (Karpman, 2012). At this point the patient can experience bloody diarrhea. The disease is further progressed with systemic spread of bacterial virulence factors into the circulatory system.

In the circulation, Shiga toxin binds on Paneth cells of intestinal epithelium by Gb3 (Globotriaosylceramide) receptors. They start to cause cell damage and even cell death in the process of apoptosis. This reduces the host immune response in the intestine. At this point the opportunistic pathogens living in the gut get a chance to do even more damage to the host (Karpman, 2012).

STEC virulence factors like Shiga toxin, LPS, circulate in the blood and bind to platelets, monocytes and neutrophils. Platelet activation leads to severe cell injury. Activation of leukocyte release proteases that also damage the organs. Microparticles are derived from both platelet and leukocytes and they bear tissue factor and that also call cell and organ damage (Karpman, 2012). Thus, endothelial cell injury, blood cell activation, other bacterial virulence etc. may contribute to severe damage and death.

2.5.1 Salmonella Typhi

Salmonella typhi cause the life-threatening disease typhoid. WHO estimates the global typhoid fever disease burden at 11-20 million cases annually, resulting in about 128 000–161 000 deaths per year (WHO, 2021). Typhoid fever causes alone cause 5% ormore of deaths in areas of high transmission (Everest, 2001).

It is usually spread through contaminated food or water. Once *Salmonella typhi* bacteria are eaten or drunk, they multiply and spread into the bloodstream.

Salmonella has a seasonal dynamic. It is observed that, peak period of this disease occur during June and September. A study was done to determine the seasonal pattern of typhoid and the data reveals such information (Saad, 2018).

2.5.2 Typhoid

Human body is the only host of *Salmonella typhi*. Infected individuals carry the bacteria in their bloodstream and intestinal tract and often develop serious symptoms. There are several symptoms that mark the infection. Such as,

- Prolonged high fever (Everest, 2001),
- Fatigue,
- Nausea,
- Abdominal pain,
- Constipation or Diarrhea (WHO, 2021).

There is a certain mechanism about how typhoid manifests. After entering the host, *Salmonella* first cause infection via the Peyer's patches (PP) and resident macrophages of the small intestine. From there, bacteria migrate into the mesenteric lymph nodes and multiply there. After a certain time bacteria are released into the bloodstream and they disseminate widely. This phase is known as the transient primary bacteremia (Everest, 2001).

S. typhi is removed from blood by macrophages that line the sinusoids of the liver, spleen and bone marrow. In these sites they can again replicate. From these sites, the re-entry of bacteria into the bloodstream can cause clinical disease. This situation is known as the secondary bacteraemia. Further, S. typhi infection of the gall bladder can lead to reinfection of the intestinal tract. At this pint serious inflammation take place and result in, ulceration and necrosis (Everest, 2001).

Microscopic histopathology of typhoid infection shows that PPs and solitary lymphoid follicles are swelled. This occurs due to the accumulation of macrophages and lymphocytes. In the tissue surrounding the gut wall, an inflammation of similar kind is seen. In the liver, spleen and bone marrow, typhoid nodules are seen. These are often comprised of macrophages and lymphocytes with central necrosis (Everest, 2001).

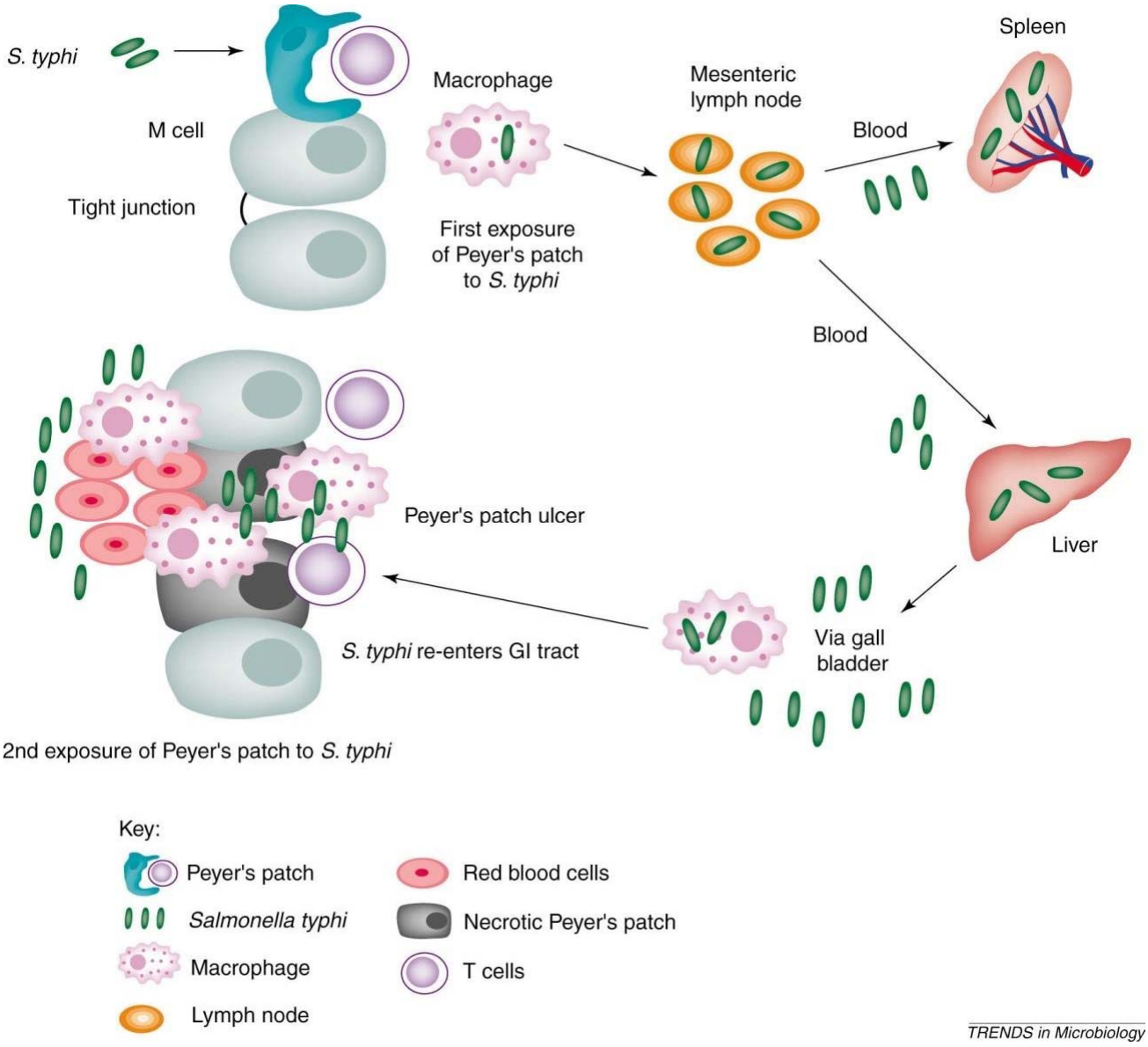


Image 2.12: Typhoid Pathogenesis (Everest, 2001)

During the third week of illness, hemorrhage from the typhoid ulcers is usually seen. Perforation of the PP can cause generalized peritonitis (swelling and redness on the belly) and septicemia (Everest, 2001). This is the most common cause of death in typhoid fever.

2.6 ELISA:

In this experiment, the optical density (OD) was measured using an Enzyme Linked Immunosorbent Assay (ELISA). Micro ELISA autoreader technique is an effective way of measuring the OD of a biofilm (Mosharraf, 2020).

ELISA is a widely used procedure in almost every immunology lab. It depends on the principle of antigen- antibody interaction. This interaction can then be quantified using ELISA Auto reader machines by measuring the OD. Substances like peptides, proteins, antibodies, and hormones can be identified and measured using ELISA. ELISA has many other names and derivations like EIA, RIA, ELISPOT (Lequin, 2005) etc. However there are three main types of ELISA. Those are:

- Direct ELISA
- Indirect ELISA
- Sandwich ELISA

In this experiment none of these techniques were used. Only the OD measuring property of ELISA Auto reader was used to get the OD of the biofilms formed inside ELISA plates

3. Materials and Methods:

3.1 Organisms:

For conducting the study, six bacterial strains were selected and tested under different conditions. 4 of them were stains of *Vibrio cholerae*. The other two were STEC and *Salmonella typhi*. The *Vibrio cholerae* strain wt-324 was used as the positive control for biofilm formation and breakdown study. Bacteria that do not form biofilms were not used in this experiment. Because the aim of this study is to examine the effects of sunlight on static biofilms. Thus, non-biofilm forming organisms is not included.

List of organisms tested:

	Organism	Attributes
1.	*Vibrio cholerae* wt- 324	Positive Control, Moderate Biofilm forming strain
2.	*Vibrio cholerae hapR*-1877	Heavy Biofilm forming strain
3.	*Vibrio cholerae hapR*-1773	Heavy Biofilm forming strain
4.	*Vibrio cholerae luxO (-)* 1712	Light Biofilm forming strain
5.	STEC *blfs 01*	Biofilm forming strain
6	*Salmonella typhi blfs 01*	Biofilm forming strain

Table 3.1: Organisms that were used in the study.

The *Vibrio cholerae* wt- 324 was used as the control as it forms a moderate biofilms. Other three *Vibrio cholerae* species, both heavy and light biofilm forming were also tested. Other two species that also cause disease in the same season were also subjected to study.

3.2 Bacterial Culture Media:

LB broth and LB Agar media was used in this experiment. All the organisms here are gram negative bacteria and LB is well suited for their growth. Other than that T1N1 media was used as preservation media. Bacterial stocks were kept in that and covered by glycerol.

All the cultures and media were taken from Life Science Laboratories, BRAC University. They were revived, used and maintained using standard protocols.

3.3 Overview of the Methods:

A protocol was developed to conduct the study and that was followed all the time. This involves every step from reviving the bacterial stock to the analysis of result.

In the beginning the strains were revived from the preservation media and made into active culture. After that, fresh culture is inoculated to fresh liquid media from plates and made into young culture by shaker incubator. Then after optimum cell density is obtained, the young culture is placed in vial/ ELISA plate to form static biofilms.

When the biofilms are ready, they were divided into two sets. One of them was and exposed to the sunlight and the other in the dark for the same time. After that the results are observed by staining in the first phase, followed by ELISA in the second phase (Mosharraf, 2020). This procedure was performed from beginning to end at least two times.

The workflow of this study is following:

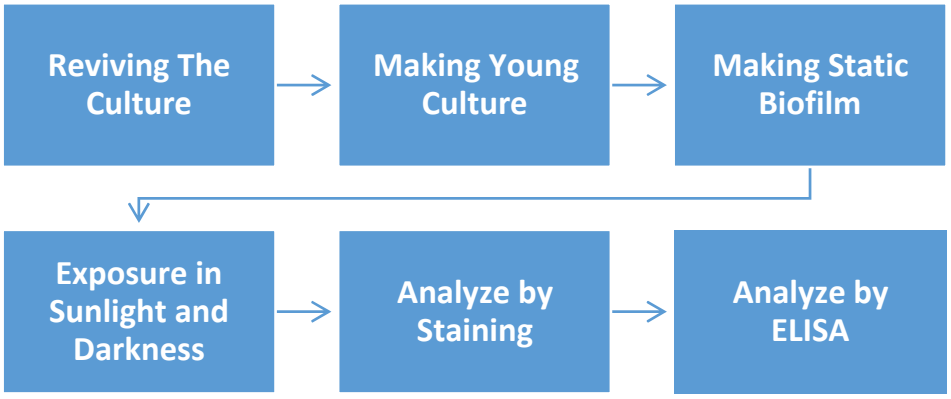


Image 3.1: Workflow for the study

3.4 Revival of Bacterial Culture:

Bacterial strains were revived from laboratory stocks, which were preserved in T1N1 Media. Culture was taken from there and revived by sub culturing on LB agar plates by streaking method. After 24 hours of incubation in 37°C temperature incubator, single colonies were isolated from the plates.

3.5 Making Young Culture and Biofilm:

For making a young culture, single bacterial colonies were taken from the plates and inoculated in 10 ml fresh LB Broth. They were cultured in 37°C and 80 rpm shaker incubator for 3 to 3.5 hours. After this period, the young culture was transferred in sterilized glass vials or ELISA Plate wells as per the study design.

If the young culture is left undisturbed for 48- 72 hours a good biofilm forms that can be seen well on the surface. For this experiment, biofilm was allowed to form for 60 hours.

3.6 Exposure in Sunlight and Darkness

Phase 01

In the first phase, 60-hour biofilms, made inside glass vials were exposed in sunlight (SET 1- S) and darkness (SET 2- D). Each SET contains 4 vials of each of the six bacterial biofilms. Everyday SET 1- S was exposed to direct sunlight for 6 hours. This was continued for 3 days in a row. After each day one set of 6 vials was stained and observed. Thus, the study was done for 3 days and 18 hours of sunlight exposure. At the same time, SET 2- D was kept away from the sun, inside a cupboard. SET 2-D was also observed simultaneously.

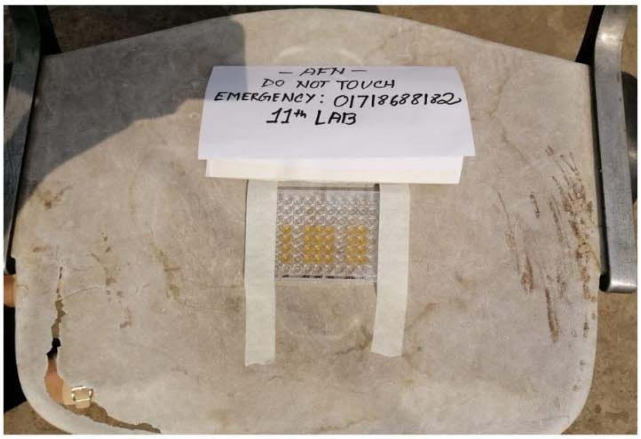
Phase 02:

After getting positive results from phase 1, the second phase was done. This time the biofilms were made in sterile ELISA plate. Two ELISA plates were made containing the biofilms. On each plate four replicates of the same biofilm was made. So that when taking the reading, an average value of optical density can be derived.

PLATE 1-S was exposed to sunlight for 6 hours a day, up to 12 hours. On the second day the

biofilms were carefully replenished with a very little amount of fresh LB Media (100 µl) just to prevent drying of the biofilm. PLATE 2-D was kept in darkness at that time. They were studied using MultiscanEX ELISA Machine.

(a) culture condition for vials in sunlight



(b) culture condition for ELISA Plate in sunlight

Image 3.2: Culture Condition of both (a) Phase 1 (Vials) and (b) Phase 2 (ELISA Plates) in Sunlight. Phase 1 was continued for 18 hours (in 3 days) and Phase 2 for 12 hours (in 2 days) in direct sunlight

3.7 Biofilm Staining:

The biofilms of both sets were observed by staining with Coomassie brilliant Blue G dye. All the samples were washed gently with sterile normal saline (0.9N NaCl), dried and stained with dye overnight. Next morning samples were again washed with saline and biofilms were observed.

Stages of Biofilm Observation by Staining

Stages	SET 1-S (Exposed to Sun)	SET 2-D (Kept in Darkness)
Stage 1	Before Exposure (vial 1)	Before keeping in Dark
Stage 2	After 6 hours in the sun (vial 2)	Same time as (SET 1-S, vial 2)
Stage 3	After 12 hours in the sun (vial 3)	Same time as (SET 1-S, vial 3)
Stage 4	After 18 hours in the sun (vial 4)	Same time as (SET 1-S, vial 4)

Table: 3.2: Stages of Biofilm Observation by Staining

The Biofilms were observed at 4 stages. Once before exposure, and after 6 hours, 12 hours and 18 hours of gradual sunlight exposure

3.8 ELISA of Biofilms:

MultiscanEX ELISA Machine by Thermo Scientific was used to measure the absorbance of the biofilms. The Biofilms from both the plates were measured 3 times, at 450 nm wavelength.

Stages of Biofilm Observation by ELISA

	PLATE 1-S (Exposed to Sun)	PLATE 2-D (kept in darkness)
Stage 1	Before Exposure	Before keeping in Dark
Stage 2	After 6 hours in the sun	Same time as PLATE 1-S
Stage 3	After 12 hours in the sun	Same time as PLATE 1-S

Table 3.3: Stages of Biofilm Observation by ELISA

The Biofilms were observed at 3 stages. Once before sunlight exposure. And after 6 hours and 12 hours of gradual sunlight exposure.

4. Results:

4.1 Result of Staining:

After exposure in sunlight periodically for 18 hours, a gradual degradation of biofilms was observed in each glass vial. For each of the organism, the result was pretty similar. Each day the biofilm becomes thinner and thinner. This suggests that sunlight could be an effector of biofilm degradation.

At the same time the biofilms kept away from light show no significant change. They remain the same after 18 hours without any significant sign of degradation. This also strengthens the idea that, maybe sunlight is quiet responsible for degradation of biofilms.

Result of Staining for all six specimen:

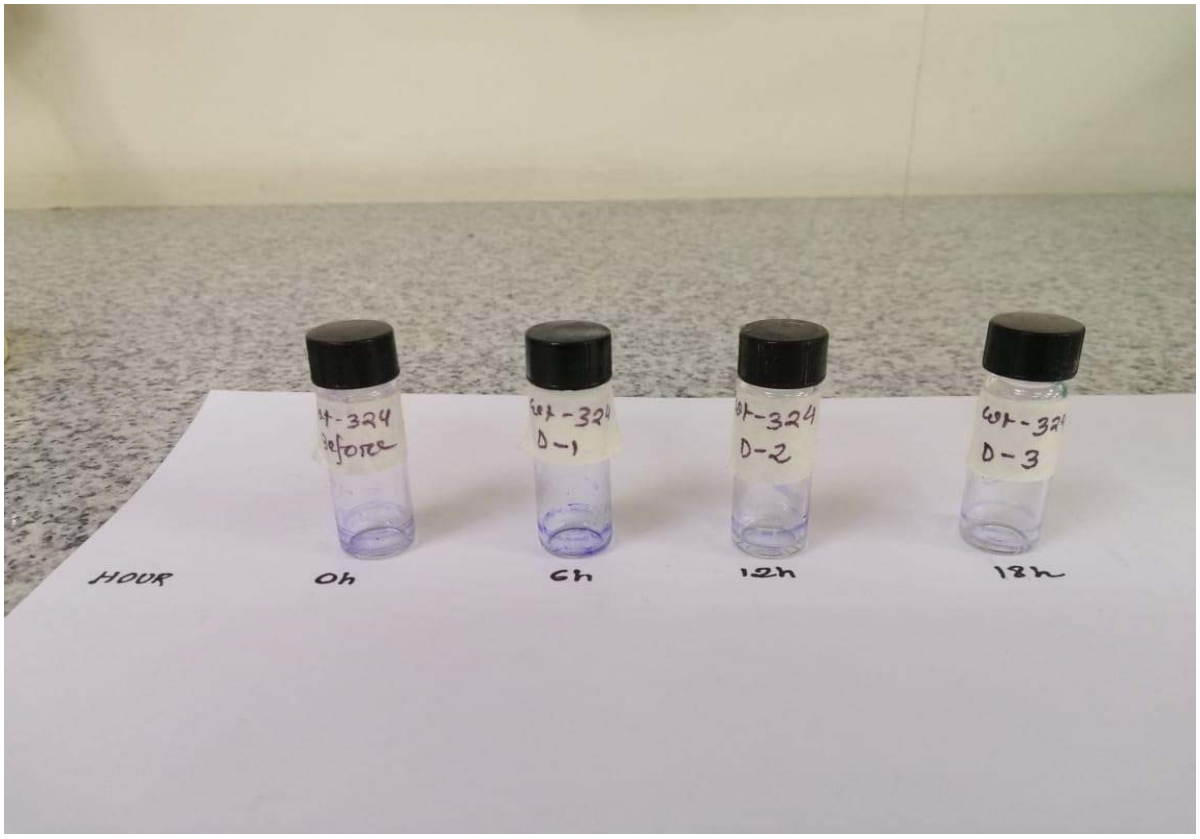


Image 4.1: Staining of *V. cholera wt-324* biofilms in sunlight upto 18 hours

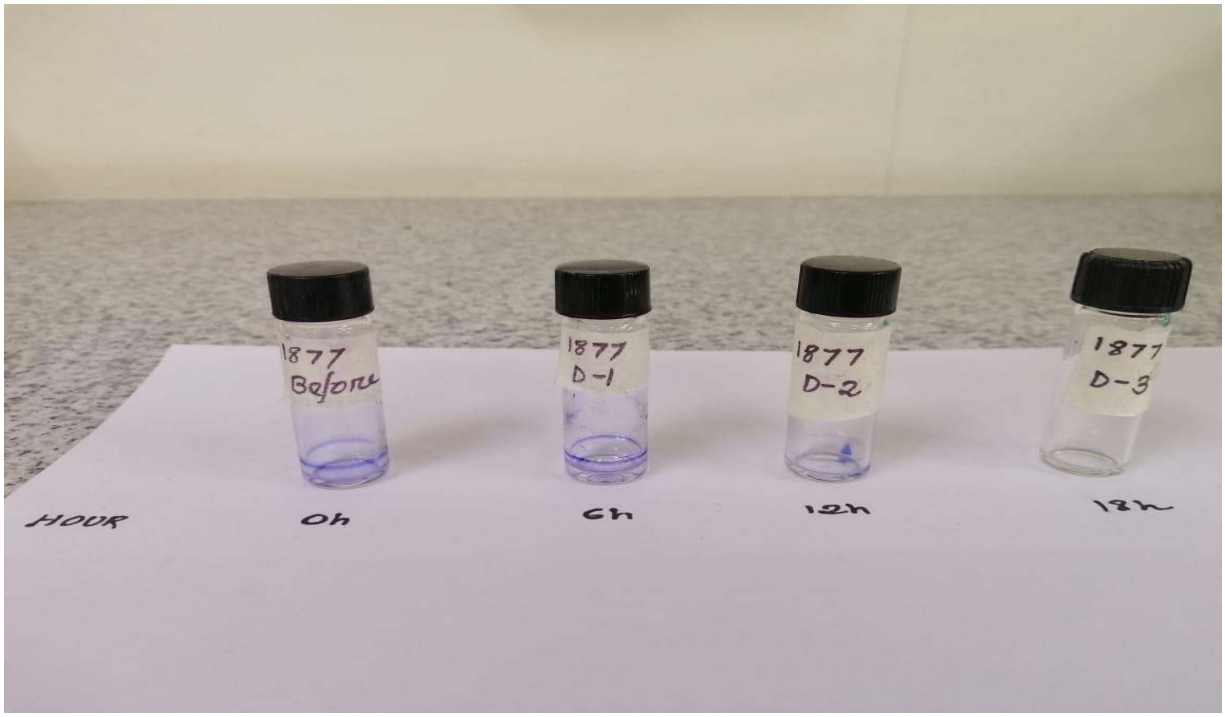


Image 4.2: Staining of *V. cholera hapR 1877* biofilms in sunlight upto 18 hours

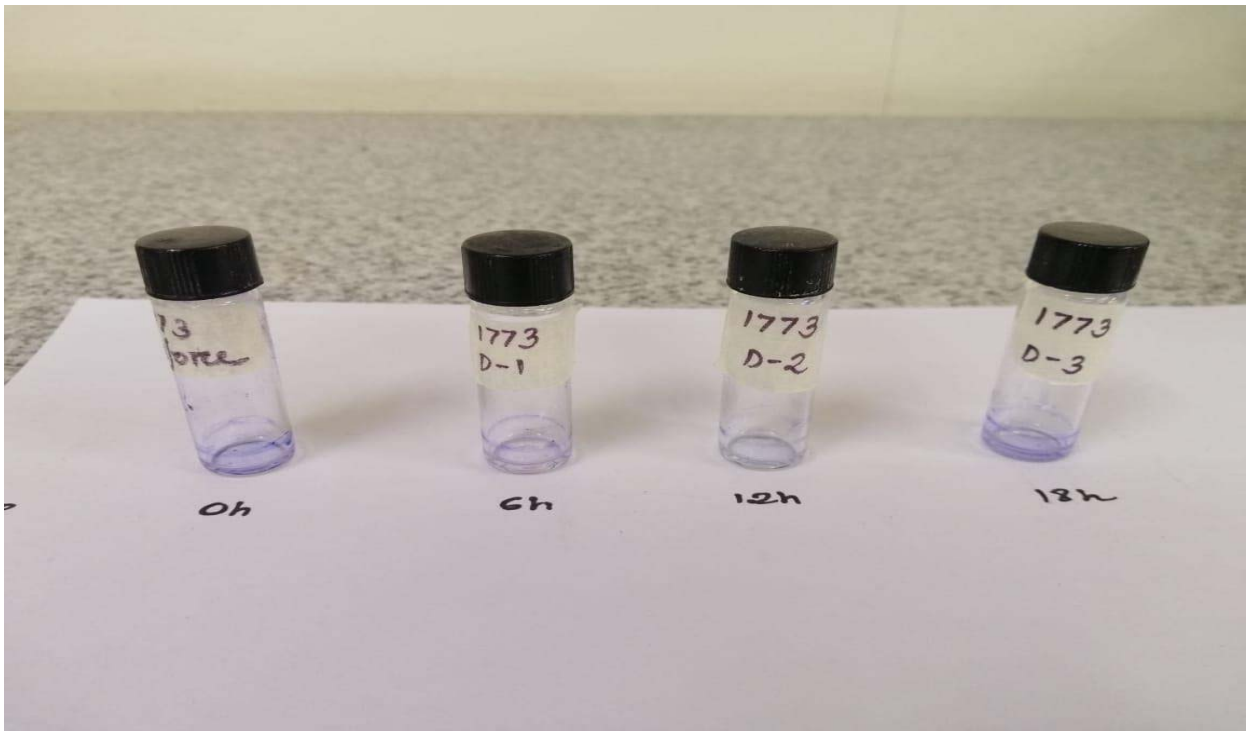


Image 4.3: Staining of *V. cholera HapR 1773* biofilms in sunlight up to 18 hours

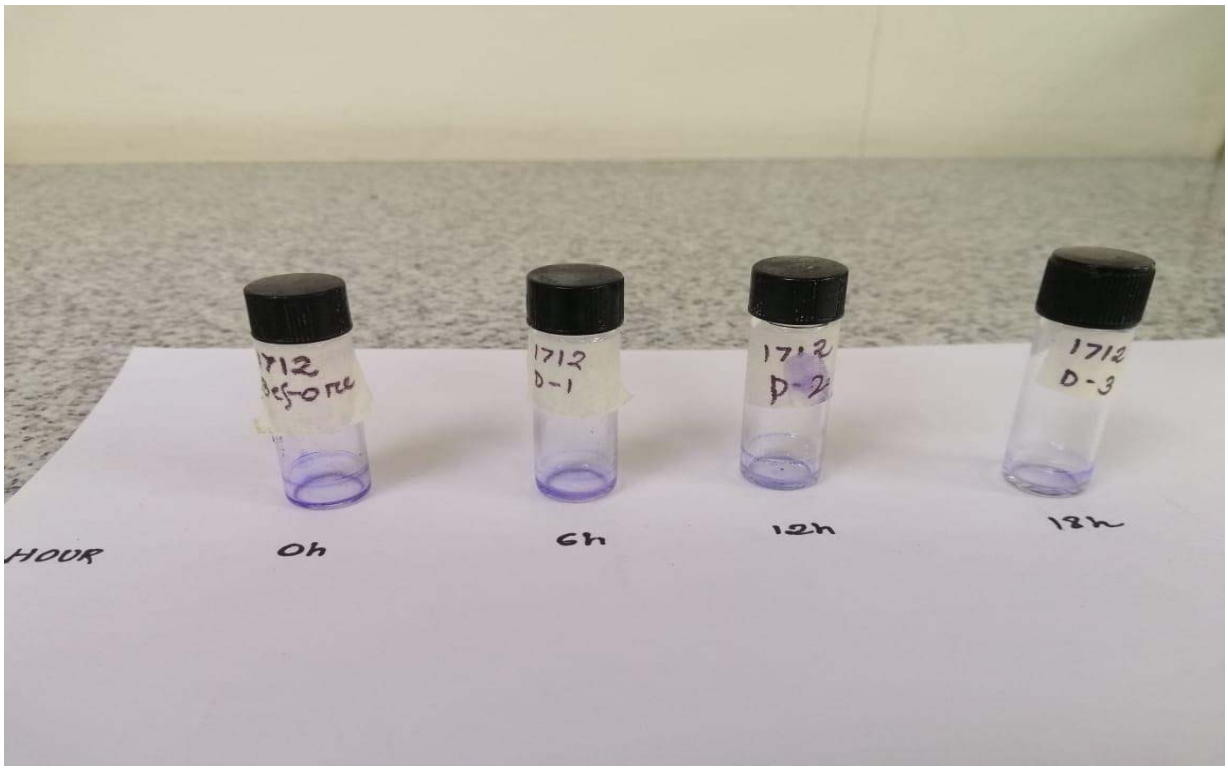


Image 4.4: Staining of *V. cholera LuxO- 1712* biofilms in sunlight up to 18 hours

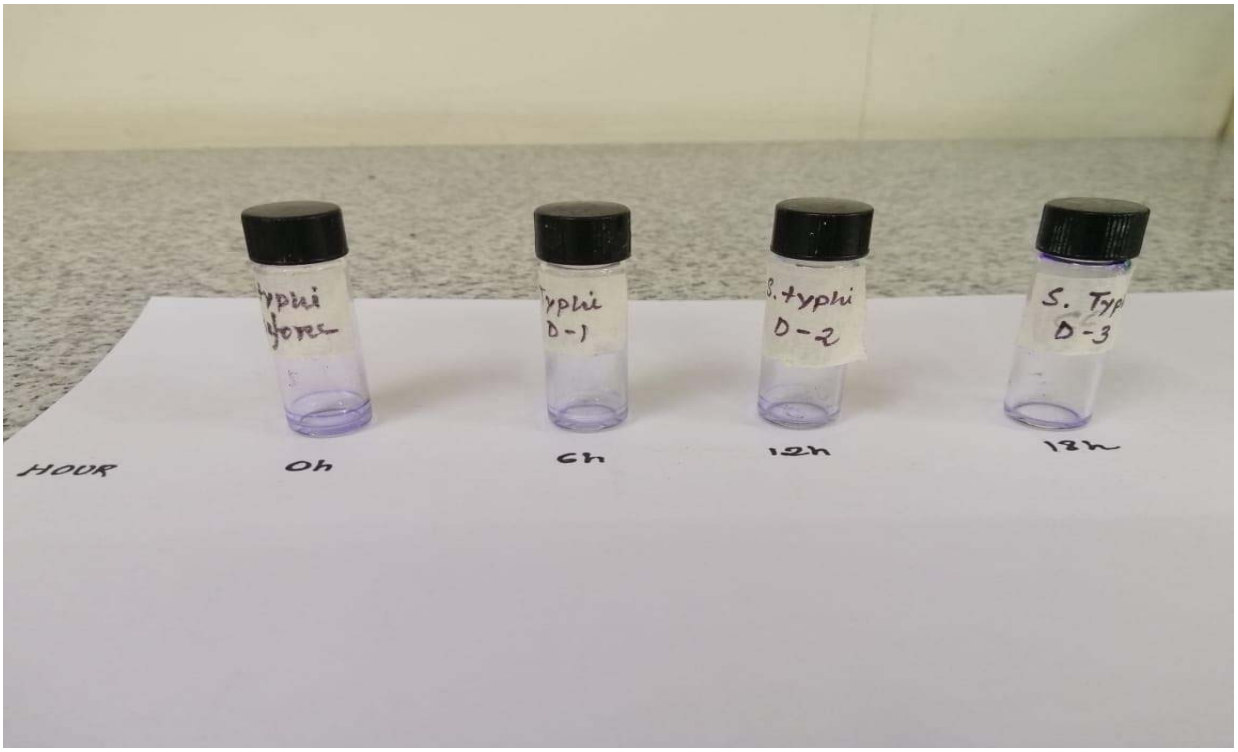


Image 4.5: Staining of *Salmonella typhi blfs 01* biofilms in sunlight up to 18 hours

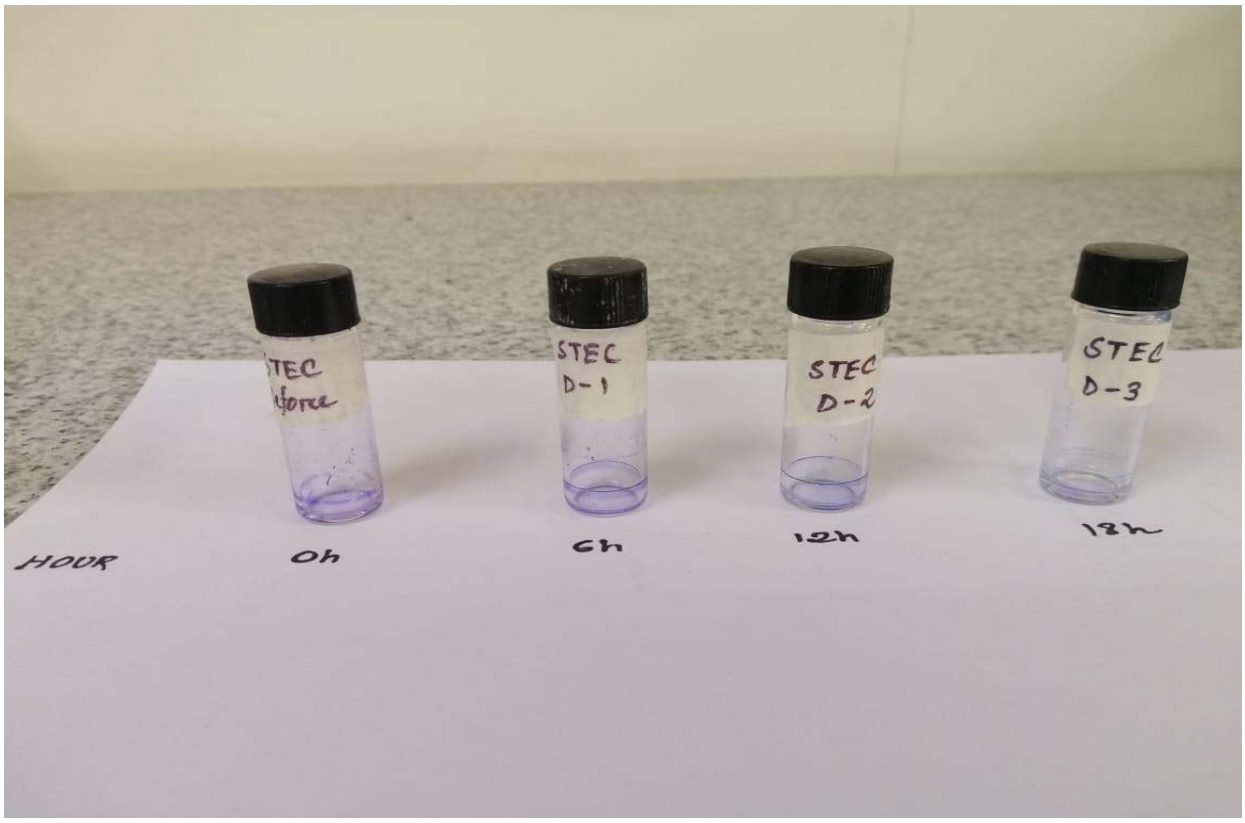


Image 4.6: Staining of STEC *blfs 01* biofilms in sunlight up to 18 hours

These six pictures show six different organisms used for this study. In each pictures there are 4 vials. They all contain the biofilms that were exposed to sunlight consecutively every day for 0 Hours, 6 Hours, 12 hours and 18 hours. And a gradual degradation of biofilms is seen in almost every vial.

4.2 Results of ELISA:

After getting convincing results from staining tests, the study was moved to the second phase. ELISA was used to directly measure the optical density of the biofilm formed over the culture medium, kept in both sunlight and darkness for up to 12 hours. It is to be noted that all biofilms were kept at a similar temperature. One ELISA plate was exposed to the direct sunlight, the other one kept coved inside a cupboard to cancel all kinds of light.

4 replicates of each organism were made in both the ELISA Plates. The plates were measured at regular intervals by the ELISA Machine.

ELISA Results (Optical Density) for biofilms exposed to Sunlight

Organism/Well	Well-1			Well- 2			Well-3			Well-4		
	0H	6H	12H	0H	6H	12H	0H	6H	12H	0H	6H	12H
Vibrio Cholerae wt- 324	0.98	0.10	0.34	0.90	0.29	0.24	0.97	0.14	0.25	0.86	0.25	0.09
Vibrio Cholerae hapR 1877	1.29	1.13	0.24	1.07	0.91	0.19	1.05	0.92	0.23	1.08	0.83	0.20
Vibrio Cholerae hapR 1877	0.96	0.90	0.41	0.86	0.90	0.49	0.83	0.97	0.16	0.96	1.00	0.41
Vibrio Cholerae luxO (-) 1712	0.89	0.41	0.23	0.86	0.39	0.14	0.81	0.40	0.12	0.85	0.39	0.19
STEC *blfs 01*	1.21	0.81	0.50	1.21	1.21	0.16	1.19	1.08	0.51	1.15	0.51	0.45
S. typhi blfs 01	0.68	0.45	0.28	0.68	0.53	0.23	0.66	0.50	0.25	0.67	0.23	0.33

Table 4.1: Optical Density Results for biofilms exposed to Sunlight under ELISA, 450 nm, H represent Hours in this table.

Such another ELISA Experiment was done for the organisms in darkness. The results are following:

ELISA Results (Optical Density) for biofilms in the Darkness

Organism/Well	Well-1			Well- 2			Well-3			Well-4		
	0H	6H	12H	0H	6H	12H	0H	6H	12H	0H	6H	12H
Vibrio Cholerae wt- 324	1.06	1.08	1.78	0.96	1.01	1.01	0.85	0.92	1.28	0.93	0.93	1.02
Vibrio Cholerae hapR 1877	1.31	1.37	1.71	1.06	1.11	1.31	1.07	1.13	1.38	1.20	1.26	1.49
Vibrio Cholerae hapR 1877	0.97	1.01	1.51	0.94	1.00	1.37	0.97	1.03	1.39	0.98	1.02	1.44
Vibrio Cholerae LuxO (-) 1712	0.96	0.98	1.18	1.58	1.62	1.91	0.82	0.84	1.06	0.98	1.01	1.12
STEC *blfs 01*	1.14	1.17	1.35	1.20	1.21	1.44	1.18	1.18	1.36	1.96	1.20	1.41
S. typhi blfs 01	0.75	0.84	1.05	0.87	0.99	1.26	0.72	0.83	0.96	0.75	0.88	1.19

Table 4.2: Optical Density Results for biofilms in the Darkness under ELISA, 450 nm, H represent Hours in this table.

After getting the values from these experiments, an average value was determined from the four readings for each organism. Error bars with standard deviation was also determined. These values were then plotted in a diagram to get a quantitative view of the biofilms degradations due to sunlight. It was seen that in all of the cases biofilms degrade with sunlight.

Average of ELISA Results (Optical Density) for biofilms exposed to Sunlight

Organism/Time	O Hours	6 Hours	12 Hours
Vibrio Cholerae wt- 324	0.964	0.893	0.277
Vibrio Cholerae hapR 1877	1.123	0.954	0.217
Vibrio Cholerae hapR 1877	0.960	0.906	0.373
Vibrio Cholerae luxO (-) 1712	0.857	0.433	0.175
STEC *blfs 01*	1.196	0.946	0.411
S. typhi blfs 01	0.676	0.443	0.2755

Table 4.3: Average of Optical Density for biofilms in Sunlight under ELISA, 450 nm

Such another average was done for the organisms in darkness. The results are following:

Average of ELISA Results (Optical Density) for biofilms kept in Darkness

Organism/Time	O Hours	6 Hours	12 Hours
Vibrio Cholerae wt- 324	0.956	1.017	1.123
Vibrio Cholerae hapR 1877	1.164	1.221	1.473
Vibrio Cholerae hapR 1877	0.967	1.022	1.433
Vibrio Cholerae luxO (-) 1712	1.091	1.115	1.321
STEC *blfs 01*	1.181	1.189	1.393
S. typhi blfs 01	0.780	0.892	1.120

Table 4.4: Average of Optical Density for biofilms kept in under ELISA, 450 nm

4.3 Quantitative Analysis of Results:

A visual representation was done to interpret the ELISA results more clearly. The results show that the concentration of biofilm over media declines with time of exposure to sunlight. The

show a direct correlation. The more the biofilm is exposed, the more the optical density drops.

On the other hand, for the plate that was kept in the darkness, the optical density of biofilms do not change that much. The slightly increase in OD value, most likely because of the presence of nutrient media and less environmental stress.

For better understanding the results of the Vibrio species are grouped together. And the result of the STEC and Salmonella are shown together. In all the images, it is observed that the longer it is exposed, the more the OD decrease, which means the biofilm, breaks down. But for the biofilms in the dark, so such decline was observed.

ELISA Results of the *Vibrio* organisms exposed to the sunlight and kept in darkness:

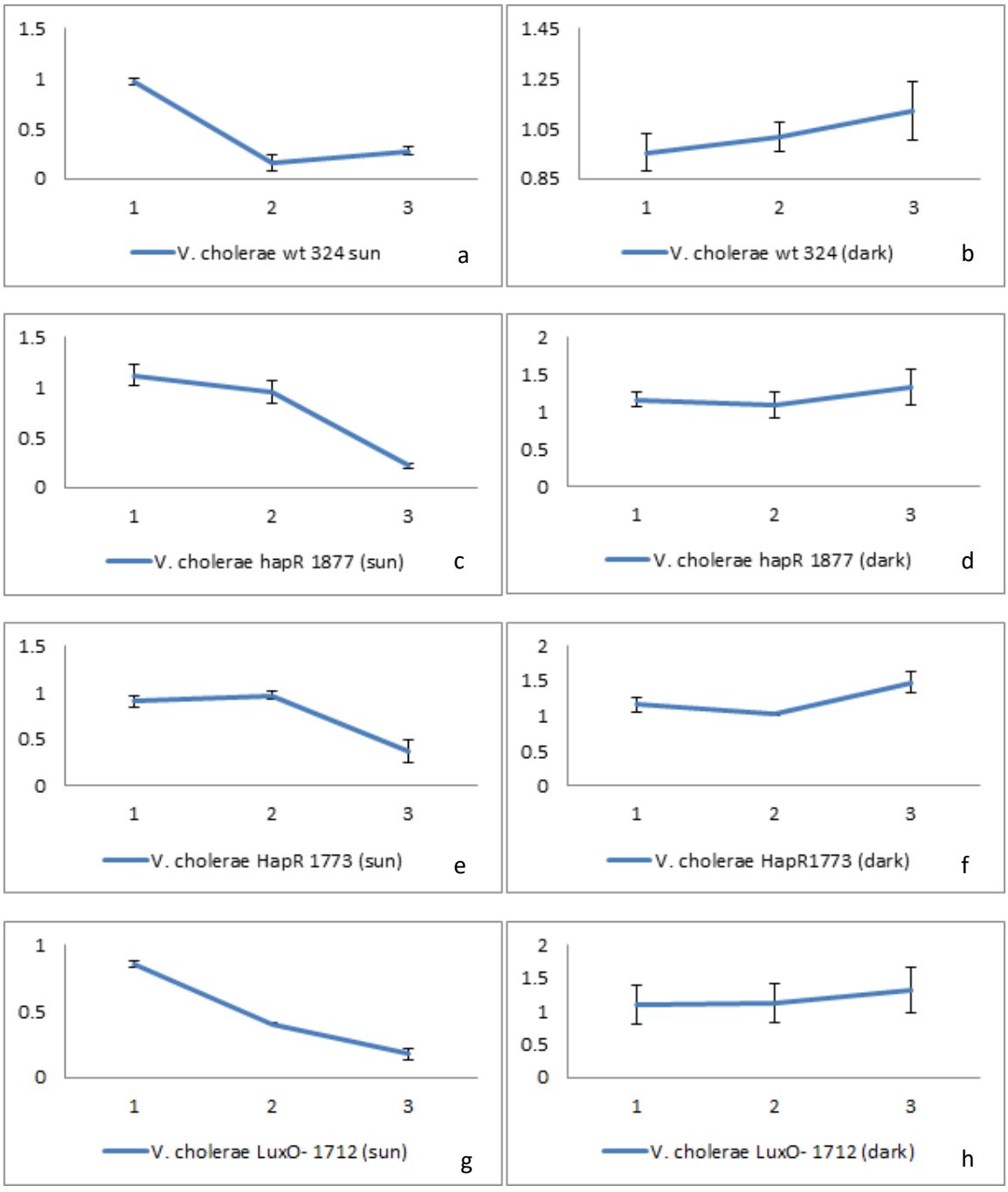


Image 4.7: Mean Optical Density (OD) in ELISA of different biofilms of *Vibrio* species. Image (a, c, e, g) represent the data in sunlight and (b, d ,f, h) represent data in darkness. Here X axis represents OD and Y axis represents the time in each image (a- h). 1, 2, 3 represent three readings at 0Hours, 6 Hours and 12 Hours in each of the image (a-h)

ELISA Results of the STEC *blfs 01* and *S. typhi blfs 01* exposed to the sunlight and darkness:

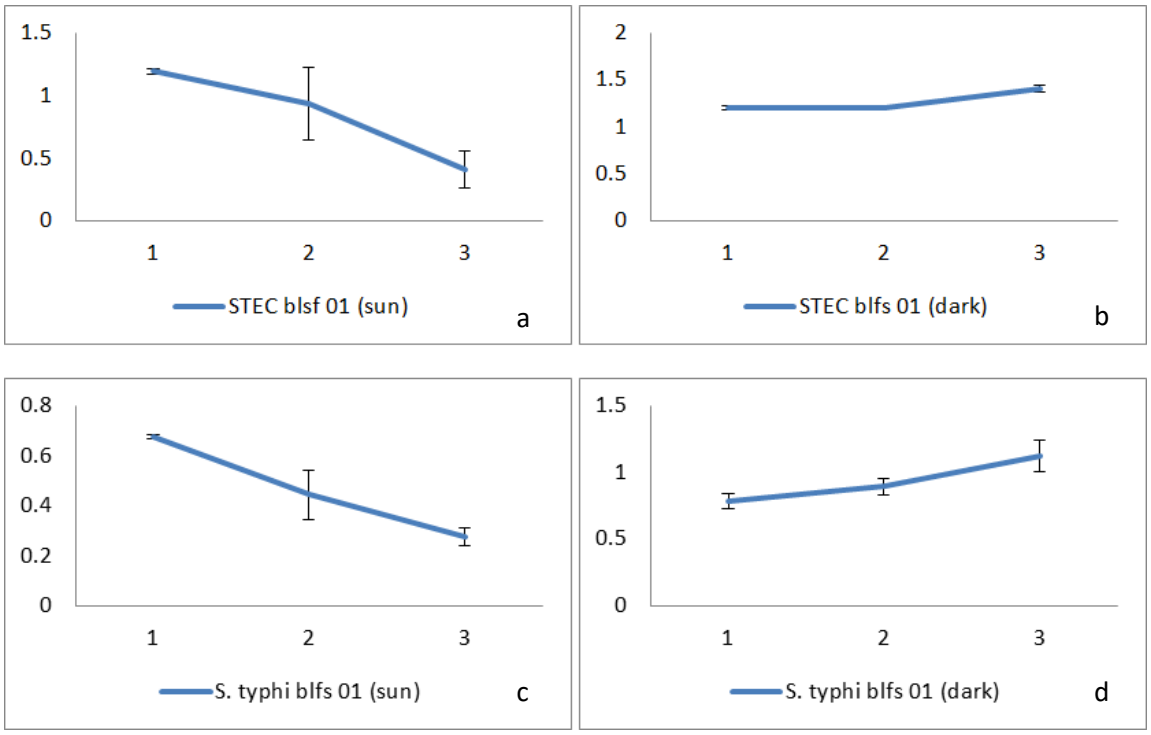


Image 4.8: Mean Optical Density (OD) in ELISA of STEC *blfs 01* and *S. typhi blfs 01* biofilms. Image (a, c) represent the data in sunlight and (b, d) represent data in darkness. Here X axis represents OD and Y axis represents the time in each image (a- d). 1, 2, 3 represent three readings at 0Hours, 6 Hours and 12 Hours in each of the image (a-d)

To summarize the data derived from these analysis, Image 4.7 (a, c, e, g), Image 4.7 (b, d, f, h), Image 4.8 (a, c), and Image 4.8 (b, d) were grouped together and shown in four different graphs. This image overall summarizes the effect of sunlight and darkness on biofilm of test bacteria.

Result of sunlight exposed *Vibrio* strains

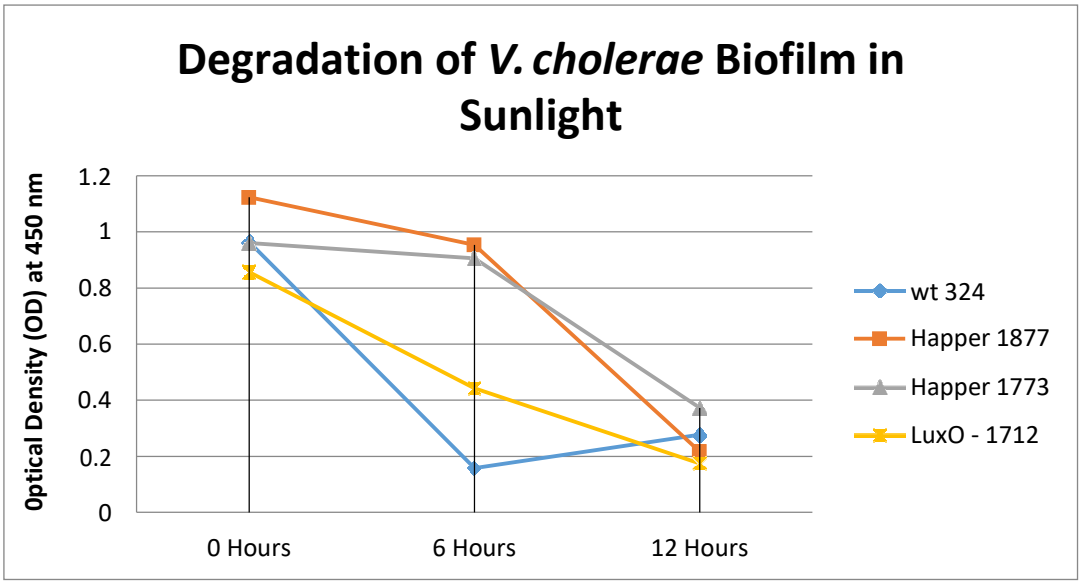


Image 4.9 : Relation of different *Vibrio* Biofilms with sunlight.

Result of *Vibrio* strains in the dark:

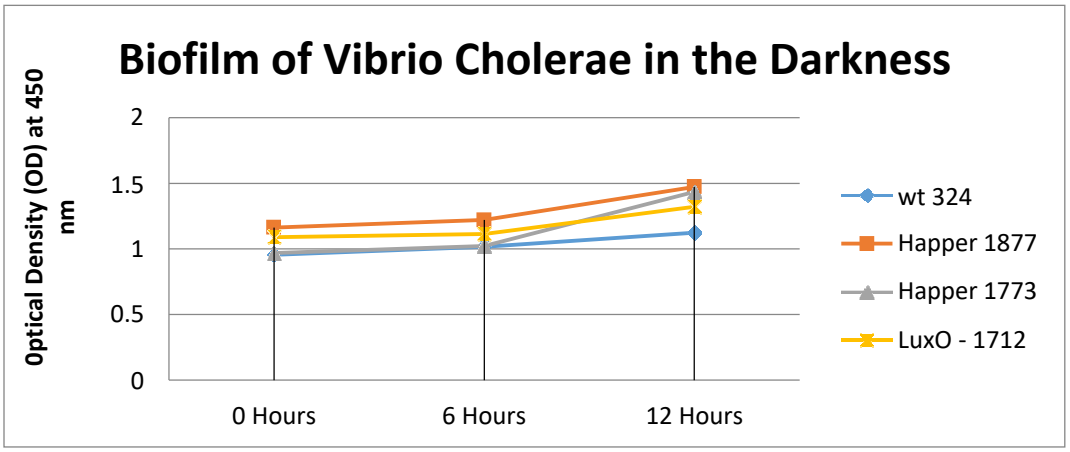


Image 4.10: Relation of different *Vibrio* Biofilms with darkness.

Result of sunlight exposed *Salmonella typhi* and STEC

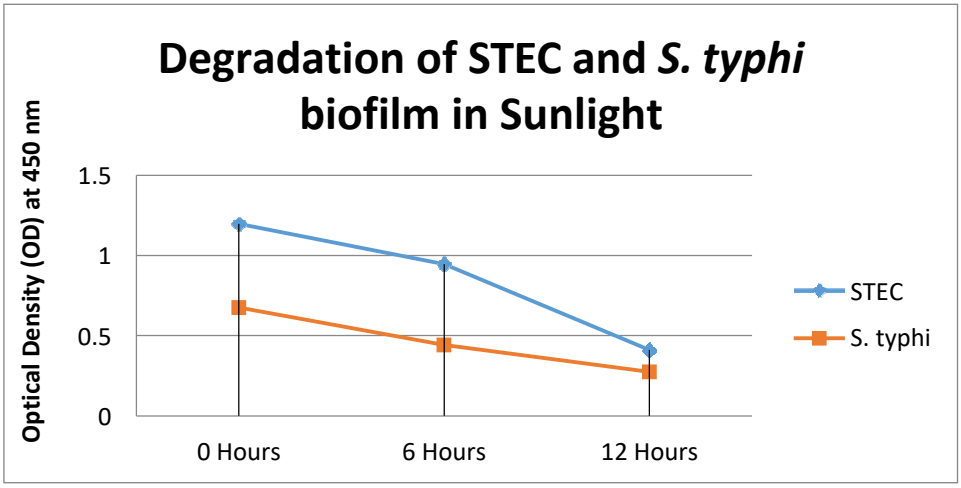


Image 4.11: Relation of STEC *blfs 01* and *Salmonella typhi* *blfs 01* Biofilms with sunlight.

Result of STEC and *Salmonella typhi* strains in the dark

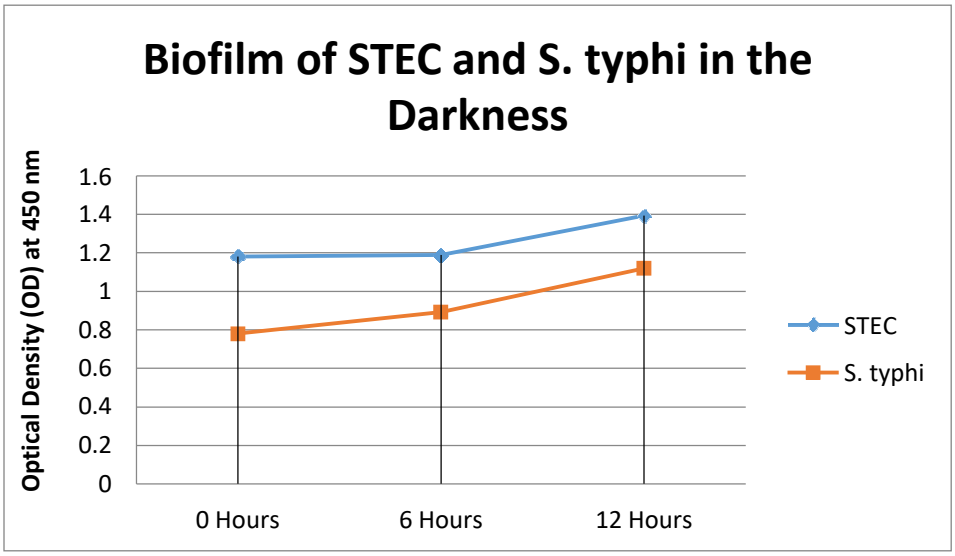


Image 4.12: Relation of STEC *blfs 01* and *Salmonella typhi* *blfs 01* with darkness.

As all the data show similar and coherent results, it can be assumed that sunlight has an effect on biofilm degradation of seasonal pathogenic bacteria. This ultimately leads to more planktonic bacteria, which give rise to more disease during the season.

5. Discussion:

5.1 Importance of Biofilms in Understanding Pathogenesis:

There are many studies that suggest a strong link between biofilm dynamics and disease pathogenesis. It is seen in many cases that, free living bacteria fail to cause a disease but when the same bacteria is in a biofilm, it becomes dangerous. Biofilm's roles have been proven in enteropathogenic infections, biliary tract infections, oral infections, ophthalmic infections, and many nosocomial infections from indwelling medical devices (Parsek, 2003). The role of biofilm dynamics in such diseases needs to be studied well for its clinical importance.

There are many advantages that bacteria gain from biofilms. For instance, in the aquatic environment, biofilms facilitate the transmission of *Vibrio cholerae* by providing a stable, protective environment and working as a hub for large number of microbe dissemination (Stoodley, 2005). To cause a symptomatic infection of cholera the infectious dose is 10^4 to 10^6 bacterial cells (Stoodley, 2005). Biofilms can be the source of this large number of bacteria in the waters during the season. Scientists tried to find out if there is any interepidemic animal carrier of *Vibrio cholerae* (Parsek, 2003). As no such animal was found, it became certain that protective biofilms in the natural aquatic environment is essential for the pathogen's survival (Parsek, 2003). Again, Biofilms can give certain pathogens selective advantage over the other at a certain season (Stoodley, 2005). That can also play a role is seasonal emergence.

Bacteria in biofilm are many times more resistant to environmental and antibiotic stress than its free planktonic form (Parsek, 2003). This resistance is certainly is a matter of investigation in case of medically important biofilms. When bacteria go into biofilm form its lifestyle changes significantly. A major switching in gene expression is expected to happen in this case (Reisner, 2005). Further study into this matter can answer many questions about clinical role of bacterial biofilms.

5.2 Conditions Maintained During the Study:

In this study we have found convincing evidences that exposure in direct sunlight can cause resuscitation of planktonic bacteria from biofilms. For the results to be precise and reliable each experiment was repeated at least 2 times. Besides, several culture conditions were maintained to get uniform results from all the experiments.

This work was done from March to June in the year 2021. In March 2020 some initial stage work was done that was giving positive results. However the study was interrupted due to the Covid-19 lockdown situation. As soon as the opportunity came, the experiment was repeated from the beginning. Again it had to stop because of the situation. Few things that are important to mention about this work are,

- Sterility was maintained from the beginning to the end to ensure that no other organisms or contamination interfere. All the equipment's were properly sterilized and all the lab work was done in Class 2 Biosafety Cabinet. While exposing to the sunlight, proper and sterile covering was used.
- All the specimens were kept at a similar temperature. The only difference in the two sets of the study is that, one was kept in direct sunlight and the other one in a cupboard, covered with a box where light cannot enter. It was also ensured that all the specimens receive direct sunlight evenly.
- Time duration of sunlight exposure was maintained very strictly. After each six hours of exposure, the culture was studied at the same time every day. The culture kept in the dark was also studied at that time. All the sets were then kept in the room. It is to be noted that continuous exposure for 12 to 18 hours was not done. Because most of the time natural sunlight does not exist for such a long period. However, that can be done in the future.
- Every time the experiment was repeated, the same time was allowed for biofilm formation. Biofilm grew thicker or thinner according to the characteristics of the species. But they did not receive different timing.
- The same culture media was used for all the organisms. Also the same volume of media was used in every case so that the same amount of nutrition is provided to all the subjects. While working, always fresh media was used to ensure the proper growth.

After maintaining all these matters properly, we have determined that direct sunlight has a resuscitation effect on sessile biofilms. We see many prospects of this study that are to be done in the future.

5.3 Future Prospects of the research

We plan to do the study further, in a more detailed setting. When we continue this study we plan to have a wider range of samples and at least 2 year time. There are things that are planned to be done in the future.

5.3.1 Wider Range of Media and Samples from Different Sources:

The sample size for this study was small. Four mutant strains of cholera from the laboratory were taken because of their different biofilm forming capacity. The other two were also laboratory stocks. They are also potential disease causing agents of the season. They were taken to see if all bacteria respond similarly to the experiment. It was observed that all of them indeed have a similar response.

It would be better if more strains of seasonal pathogenic agents could be included in the study. The strains should come from both clinical and natural sources. Natural reservoirs of such pathogens could be a potential source of samples. Clinical samples should also be included to analyze the results better. Thus it can be understood what effect sunlight has upon them.

The only media used in this experiment was LB media. It is important to see what results come if other growth mediums are used. This can be other basal or enriched media, or even sterilized water from natural sources.

If similar results are seen in all different setups, the effect of sunlight on biofilm breaking can be more confirmed.

5.3.2 More Adjusted and Wider span of time

This study was done in a certain time of the year (March- June). To get the complete picture, the study needs to be run at least for few years. Every month, for 2 to 3 weeks, this experiment needs to be done to truly understand the effect of seasonal sunlight. Then it can be certain that weather sunlight at a specific time of the year has distinct effect on bacterial resuscitation. This is to confirm if this event follows a certain pattern throughout the year.

In our country almost every month sunlight period varies. Therefore it should also be investigated if this affects the breakdown of biofilms. This study should be done in two different settings. One set should have the equal time of sunlight exposure. The other will be kept in sunlight for the maximum possible time. The differences of result from these experiments will answer the research question.

There lies a scope of making the experiment more detailed. Here, the results were analyzed every six hours. And after every six hour a significant decline in the biofilm's OD is observed. If time span was every two hours, the dynamics of biofilm breakdown could be clearer. It could be understood when the biofilm starts to break, if it speeds up and when it stops.

If the outcomes are in favor of the hypothesis, it can be said that sunlight works as a significant seasonal factor of waterborne disease outbreak, especially cholera.

5.4 Future Research:

If the hypothesis of this study is established, the study can be taken further. Then it can be studied what kind of mechanism goes on inside the biofilms when the break. If sunlight is a factor of biofilm resuscitation, there must be something common in these organisms. Probably that can be induced by light. Or there can be physical factors that accomplish this transition from sessile to motile form.

However, to make that conclusion this study must go on.

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