A Comparative study of the antibiotic resistance pattern between the natural and clinical isolates based on Whole Cell Protein Profiling by SDS-PAGE

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

Submitted by:
Tanim Islam
Student ID: 12126002
April, 2016

Microbiology Program
Department of Mathematics and Natural Sciences
BRAC University
Dhaka, Bangladesh

Dedicated
to
My Mother

DECLARATION BY THE RESEARCHER

This is to declare that the research work aggregating the results reported in this thesis entitled **"A Comparative study of the antibiotic resistance pattern between the natural and clinical isolates based on Whole Cell Protein Profiling by SDS-PAGE"** has been carried out by the undersigned under supervision of Fahareen-Binta-Mosharraf, Senior Lecturer, Microbiology program, Department of Mathematics and Natural Sciences, BRAC University. It is further declared that the research work reported here is authentic and submitted in the partial fulfillment for the degree of Bachelors of Science in Microbiology, BRAC University, Dhaka. Any reference to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

Tanim Islam

(Candidate)

Certified

(Fahareen-Binta-Mosharraf)
Supervisor
Senior Lecturer,
Microbiology Program,
Department of Mathematics and Natural Sciences,
BRAC University, Dhaka.

(Dr. Mahboob Hossain)

Associate Professor,

Microbiology Program

Department of Mathematics and Natural Sciences,

BRAC University, Dhaka

ACKNOWLEDGEMENT

First and foremost, I express my deepest gratitude to Almighty for endowing me with patience and mental power in all aspects of my life. This is the very first time that I have had the exposure to this kind of experimental research work and I am very grateful to everybody whoever has encouraged me and guided me throughout the course of these six months.

This work was completed under the guidance of my supervisor, **Fahareen-Binta-Mosharraf**, Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University. I am thankful to her for her trust and patience on me throughout the period of my thesis work. She added new directions to my thinking, and acted as a catalyst for the precision and satisfactory completion of the task.

I also express my gratitude towards **Prof. A. A. Ziauddin Ahmad**, Chairperson, Department of Mathematics and Natural Sciences, BRAC University, for his kind cooperation, active support and constant supervision. **Prof. Naiyyum Choudhury**, coordinator of Biotechnology and Microbiology program, Department of Mathematics and Natural Sciences, BRAC University for his guidance, monitoring and constant encouragement throughout the project. I would specially like to thank **Dr. Mahboob Hossain**, Associate Professor of the Department of Mathematics and Natural Sciences, BRAC University, for his immense support and cooperation.

I would also like to extend my gratitude to **Nahreen Mirza**, Teaching Assistant, Department of Mathematics and Natural sciences, BRAC University, for her support and guidance in all the laboratory tasks. I am indebted to all the Lab Officers and the Lab Assistants who had always been there to guide me.

I am thankful to all of my friends, for their help and support throughout my work. Finally I want to extend my special thanks and gratitude to all my seniors and batch mates in the microbiology and biotechnology laboratory, who have provided good working environment by sharing their advice and encouragement to make me feel at ease even in the hardest times.

Tanim Islam

April, 2016

Abstract

The comparative study was executed to detect and examine the presence of antibiotic susceptible and antibiotic resistant bacteria in several environmental samples of Bangladesh. A specific clinical strain was obtained from ICDDR,B. Numerous environmental samples were collected from different locations of Dhaka city in order to verify the consistency of the environmental strains and their pattern of antibiotic resistance. All the standard biochemical and morphological tests were conducted to confirm the bacterial strains, after the isolates were cultured in a standard selective medium. Only, one single strain was chosen for the further study. Following CLSI (Clinical & Laboratory Standards Institute) guideline, the antibiotic sensitivity of the isolates was determined by carrying out antibiogram. The follow up of the entire study shows that the clinical strains were either resistant or had intermediate effects against most of the antibiotics, while the environmental strains were either sensitive or had intermediate effects against most of the antibiotics with an exception of one or two antibiotics against which these strains were resistant. The whole study provides an idea about the future emergence and the development of more counterproductive strategies to overcome this serious problem of multidrug resistance amongst the pathogenic organisms. The research work can be extended with the development of laboratory facilities, and in near future the characterization of the antibiotic resistant proteins of these organisms can be done.

Contents

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

NA	Nutrient Agar
MAC	MacConkey Agar
CFU	Colony Forming Unit
XLD	Xylose Lysine Deoxycholate
LB	Luria Bertani
TSI	Triple Sugar Iron
NaCl	Sodium Chloride
ICDDR,B	International Center for Diarrheal Disease Research, Bangladesh
IMViC	Indole, Methyl red, Voges-Proskaur, Citrate
MHA	Mueller-Hinton Agar
FOX 30	Cefoxitin
SXT 25	Sulfamethoxazole/Trimethoprim
CIP 5	Ciprofloxacin
PEF 5	Pefloxacin
E 15	Erythromycin
CN 10	Gentamycin
S 10	Streptomycin
K 30	Kanamycin
C 30	Chloramphenicol
NA 30	Nalidixic Acid
CXM 30	Cefuroxime Sodium
OX 1	Oxacillin
F 300	Nitrofuantoin
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
BRAC	Bangladesh Rural Advancement Committee

Introduction

1.1) Overview

The comprehensive study and research on bacterial pathogenicity and the evolution of their ecology of the infectious diseases leads to the extraction of several benefits, some of which the development of theories on how infectious diseases are contagious and how the diseases can be transmitted and also treated with beneficial health effects and how the infectious diseases emerge and reemerge. The most substantial benefit could be the capacity to prerecognize the outbreaks relevant to that particular infectious disease.

It has always been observed that the host-microbe relationship is a dynamic equilibrium. The invasion of the commensal microbes within the hosts is usually triggered by either a genetic change or physiological change in either of the host or the particular microbial population. This sets in motion of an immune response from the host as a mechanism of defense against the pathogenicity and the subsequent disease caused due to the pathogenicity of the microbe. A growing interest among the community of microbiologists is to study this dynamic relationship as it is imperative to maintain our bloated life expectancy bestowed by the era of antibiotics. In this trend, common disease-causing microorganisms and our methods to eradicate or control them are subjects of repeated rigorous scientific experiments.

The evolution of the enteric disease causing pathogens throughout the history of modern medicine can be exemplified as a palpable one which makes the research work on such relevant topics more appreciated and desired. There are very few examples which claim that the infectious diseases caused by a pathogen have led to extinction of a certain species. However, treating most of the infectious diseases are becoming more diverse and complex at the same time due to the development of resistance of these pathogens against the most well applied and used drugs. In the public consciousness, a disease like diarrhea has gone from being a sweeping epidemic that leaves us mostly helpless, to a benign inconvenience that we have to endure for a few days, but this underlies an uphill battle between synthetic antibiotics and disease causing pathogens, that microbiologists have been fighting ever since the days of penicillin.

1.2) Bacteria as a pathogen

Bacteria are unicellular and one of those microbes which are the most common studied and researched one for the development of new medicine. There are thousands of different kinds, and they live in every conceivable environment all over the world. Disease causing bacteria can be found to reside in variegated areas such as deep within the earth's crust, seawater, soil and also in radioactive waste. There also many bacteria that reside within the human body and are harmless. Such bacteria are known as the normal flora or the resident flora and can cause opportunistically. Bacteria can cause diseases by invading the tissues of the host, by releasing harmful substances (toxins) or doing both.

1.3) Bacterial classification

Bacteria can be classified in several ways:

Table 1: Classification of bacteria

a) Scientific names	Genus classifies bacteria and, within the genus, the bacteria are classified by species. Their scientific name is genus followed by species (for example, *Clostridium botulinum*). Within a species, there may be different types, called strains. Strains differ in genetic makeup and chemical components.
b) Staining	Usually bacteria are classified either as Gram negative or as Gram positive because of the difference in the composition of their cell walls. Upon gram staining, the gram positive bacteria usually stain violet/purple whereas the gram negative ones stain red in color. They also cause different types of infections, and different types of antibiotics are effective against them.
c) Shapes	Bacteria can usually have one of the three types of shapes. 1) Rods (*Bacilli*) 2) Spheres (*Cocci*) 3) Spirals (*Spirochetes*)
d) Need for oxygen	Bacteria can be classified on the basis of their need for oxygen in order to grow. The aerobes are the ones which require oxygen for the growth and ones which do not require an oxygen supply are known as anaerobes. Some bacteria known as the facultative bacteria are the ones which can live and grow with or without the supply of oxygen.

1.4) Salmonellosis

Salmonellosis is popular as one of the major and worldwide food borne disease, causing severe outbreaks in the developing countries. The outbreaks can be contained and small or even large outbreaks in the general population. Immuno-compromised patients, or patients infected with HIV virus are very prone to salmonellosis. The disease is common amongst children, even though older infants and even adults can get infected. There are different types of *Slamonella* bacteria which can cause the disease. However, the most common organisms which can infect animals and humans are *Salmonella typhi* and *Salmonella entiritidis* .The disease can transmit in various ways, one of which is preparing food by unwashed hands. Moreover, poorly cooked meat or raw meat products carry a considerable load of *Salmonella* as well as raw fruits and vegetables with rough surfaces are heavily contaminated with the organism. Symptoms like high fever, diarrhea, abdominal cramps, vomiting, nausea, etc. can begin in between 12 hours to 3 days of the infection and last till 7 days. Many strains of *Salmonella spp* have developed multi drug resistance and therefore it is very necessary to consume the right antibiotic for the right amount of time in order to get fully treated if the infection penetrated from the intestine to the blood stream. However, in normal cases, oral fluids and intravenous fluids are the suggestive treatment to overcome the infection.

Infectious diseases are the physiological consequences of a numerous number of organisms which are considered to be harmful human pathogens. All these pathogens are inclusive of both the pre-existing and recently derived ones which bring out an effective change within the health of humans and can also be responsible for a humongous number of deaths every year. On the contrary, as much as infectious diseases are increasing and leading to the death rate of large human populations, studies have shown that there are also some infectious diseases that are slowly but significantly eradicating over the past few years. The re-surfacing of these declining diseases can be interpreted with the relevance of several routes of transmission and a vast spectrum of hosts. Emergence of these pathogens are intrinsically whimsical but they tend to share some general and common characteristics and therefore the straightly transmitted RNA viruses are doubted to be the pathogens that superficially plunge in between the different host species. It has been always believed that every infectious disease must have been emerged at some point and this lead to the broad and multiple research study in order to decode the epidemiology and evolution of such diseases. The field of molecular diagnostics has been provoked since years now to implement the topical knowledge to assist the progress of agent diagnosis and further exploration of the novel pathogens.

1.5) The sporadic resistance of bacteria to the antibiotics

It was not long ago when antibiotics were glorified as the wonder drugs which could be mighty enough to cure every possible death threating diseases. Satirically, with the evolution of both the infectious diseases and their subsequent treatment, significant number of antibiotics have become less cogent due to the over usage of them in treatment of large populations. The misery is that even though there are advanced antibiotics which are being derived frequently, most of them have been failed to have a long lasting effect as declared by many reports. Consequently, in the past few years an elevated level of resistance has been developed among the pathogens against the curative agents. Thus a potential harmless microbe can gradually become a harmful pathogen over a time span [2].

The myriad and chaotic dependence and usage of the commercially available antibiotics in order to cure the variegated infectious disease has been the chief reason for the development of multi drug resistance in the human pathogenic microbes which has increased over the past few years. The elements that assisted the development of such resistance has been more than one, such as the environmental factors, the characteristics and traits of the hosts, the host-pathogen specific relationship and the usage of antibacterial agents [3]. The capacity of these bacteria to come up with new ways to invade their hosts is highly influenced by the fact that these pathogens have the ability to evolve very swiftly. This favors the development of antibiotic resistance within them and is a matter of concern that the number of pathogens showing such patterns are only increasing. Also the usage of the high levels of antibiotics in animals and humans has augmented the emergence of these antibiotic resistant strains which are the core focus of this study.

1.6) SDS-PAGE

SDS refers to the chemical agent known as Sodium dodecyl sulfate. The main principle of SDS-PAGE is to separate the proteins by their size and according to no other physical feature. The electrophoresis technique here uses a discontinuous polyacrylamide gel and the denaturing agent SDS to bring out the separation.

1.7) SDS

In order to achieve the separated protein fragments of different sizes, the prior denaturation of the proteins is a major requirement of the process in order to get the protein in its linear form with its secondary, tertiary and quaternary structures being totally nonfunctional.

As the name of the process suggest, the electrophoresis technique relies on the usage of an electric field for the separation of the macromolecules whereas the polyacrylamide gel acts as the support medium.

SDS is an anionic detergent which charges its entire dissolved molecule with a net negative charge with a wide range of pH. The binding of a polypeptide chain with SDS is based on in proportion to its relative molecular mass. The negative charge on SDS neutralizes the most of the complex structures of the proteins and thus the proteins are strongly attracted to the positively charge electrode (anode) in an electric field.

1.8) Polyacrylamide gels

It is known that the mobility of the particles in a gel depends on both its size and charge. The mobility is also related to the frictional resistance provided by the medium. The migration of the large molecules as fast as those off the smaller ones is hindered by polyacrylamide gels. The final separation and migration rate of the SDS denatured proteins exclusively depends on the relative molecular mass of the polypeptides as the charge to mass ratio of every denatured fragment is almost the same. When a gel has uniform density, the Rf value that is the relative migration distance of a protein is negatively proportional to the log of its mass. The relationship between the Rf and mass of the known and unknown proteins can be plotted in a graph if the known and proteins of known mass are run simultaneously with the unknown ones. SDS-Page separated proteins can be utilized for various purposes such as determining the relative abundance of the primary proteins in a sample, to estimate their relative molecular masses and to resolve the distribution of the proteins among fragments. The voyage of the purification of the proteins can be gauged and followed.

1.9) Aims & Objectives

The significant treatment failures and increase expenses of health care has led to a rise in the mortality rates. It is believed that the development of antibiotic resistance in the pathogens have influenced this. Emergent antibiotic resistance one of the solemn universal problem, which is difficult to be elaborated in the context of defining the public health risks and estimation of the health costs , as these would involve vast research studies and cannot be simply undertaken. The rampant and rapid usage of the antibiotics triggers a selective pressure that acts as a catalyst to the development of antibiotic resistance.

However, the frequency in discovery and development of newer and stronger antibiotics by the pharmaceutical companies have been significantly high since a decade now, but still it is assumed and noted that there has been a simultaneous increase in the resistance against these not so old antibiotics. New therapeutic drugs and other alternatives should be developed only after proper assessment of the resistance pattern of the most common pathogens against the standard antibiotics. This would be fruitful enough to minimize the synthetic antibiotic side effects.

In accordance to the above framework, the main objectives of this study are:

i) An environmental strain of a common pathogenic organism would be isolated and confirmed for the morphology of the same organism.
ii) The degree of antibacterial activity of the natural and clinical sample would be estimated by measuring the diameter (mm) of zone of inhibition on each agar plate.
iii) The pattern of antibacterial activity of both the clinical and environmental sample would be compared.
iv) Proteins of both the clinical and environmental samples would be subjected to SDS-PAGE for separation of these proteins according to their size only.
v) Estimating the presence of unknown proteins in the environmental strain and justifying the function of it.

Chapter 2 – Materials and methods

2.1) Working laboratory

All project tasks were executed in the Microbiology and Biotechnology Laboratory, Department of Mathematics & Natural Sciences, BRAC University.

2.2) Reference Bacterial Strains

In this study, one standard clinical strain of *Salmonella typhimurium* and several environmental strains of *Salmonella spp.* were used from which one target strain was chosen. The clinical sample was obtained from ICDDR, B (International Center for Diarrheal Disease Research, Bangladesh).

2.2.1) Preparation of the bacterial plating

- 5 ml of Luria Bertani (LB) broth was prepared into a screw capped test tube.
- A single colony of the clinical strain of *Salmonella typhi*, which was already cultured in a Nutrient Agar (NA) plate was touched with a burnt loop and inoculated into the test tube containing Luria Bertani broth.
- The test tube was incubated at 37^0C in a shaking incubator (Daihan Scientific, Korea) at 120 rotations per min (rpm) for approximately 3 hours.
- After the incubation period was over, 1 ml of the culture was taken from the test tube into an eppendorf and centrifuged (Eppendorf, Germany) at 12,000 rpm for 10 minutes.
- After the centrifugation, the supernatant was discarded carefully.
- To make the bacterial pellet homogenous, it was suspended properly in 1 ml of 10M MgSO4.The suspension was stored at 4^0C until used.
- The suspensions were then diluted 1:100 in TSB broth to obtain 106 CFU/ml before use.

2.2.2) Confirmation of the reference strains

The reference bacterial strains were distinguished by sub culturing the clinical strain on recommended selective media (Table 2) and by identifying the morphology of the organism (Table 3) by conducting gram staining. The cultural properties of the organism were observed and the results were recorded.

Table 2: Respective Selective Media for Reference Strain

Reference strains	Selective media
Salmonella typhimurium	Xylose Lysine Deoxycholate (XLD) agar

Table 3: Gram staining property of the Reference Strain

Reference strains	Gram stain property
Salmonella typhimurium	Gram -ve (stains red) under the microscope

2.3.1) Environmental sample

The sources for the environmental sample were chosen to be raw fish water from local fish markets and salad from local restaurants. The samples were collected in the month of October 2015. Throughout the month of October, the temperatures at the day time would be in between 30^0C-35^0C and the temperatures at the night time would be in between 18^0C-22^0C. The average relative humidity for the whole month was around 85%.

For achieving the best environmental strain, five different salad samples and eight different raw fish water samples were collected and tested for the presence of the *Salmonella spp*. From all these samples, five different organisms were predicted to belong to the *Salmonella spp*. After conducting all the necessary identification, biochemical and morphology tests with all the five strains, the strain for which the results were identical to all the properties of the clinical strains were chosen as the test environmental strain for the study.

Table 4: Source of environmental samples

Environmental sample	Collected from
Handmade salad	Local restaurants located at Mohakhali, Banani, Dhanmondi, Mohammedpur and Mirpur.
Raw fish water	Local fish markets situated at Mohakhali, Dhanmondi, Mohammedpur, Banani , Mirpur, Gulshan , Adabor and Shukrabad.

2.3.2) Sample processing

All the environmental samples were serially diluted to six folds of dilutions following the standard sample processing technique.

- Physiological saline was prepared in sterile test tubes.
- I ml of the sample suspension was then transferred to 9 ml of physiological saline in the test tube using a micro pipette.
- The test tube was vortexed properly.
- The same procedures were followed for all the thirteen samples of the samples.
- The whole procedure was carried out under the laminar flow.

2.3.3) Assortment of desired colonies from nutrient agar plates

All the diluted samples were spread onto Nutrient agar plates for heterotrophic count. For each of the thirteen different samples, multiple colonies from each of the nutrient agar plates were

selected depending on the cultural characteristics and transferred to specific standard selective media (Xylose Lysine Deoxycholate) for further confirmation.

After spreading each of the samples on XLD agar media, and their subsequent overnight incubation, the results were observed and checked to compare with the growth properties of *Salmonella typhimurium* in XLD agar media. The colonies for each of the samples that coincided with the colony properties of *S.typhi* were later cultured into fresh nutrient agar plates and labeled appropriately. Five different strains were obtained from the assortment and the strains were labeled as Sal1, Sal2, Sal3, Sal4 & Sal5. All these five strains were subjected to biochemical tests and gram staining, and out of them Sal1 was chosen to be the test strain with its coinciding cultural and characteristic properties to that off the clinical strain.

2.4) Biochemical confirmation of the clinical strains and environmental strains

Consequently, the clinical strain and all the five environmental strains were subjected to biochemical test followed by morphological test. All the recommended biochemical tests like Methyl red test, Voges–Proskaur test, MIU (Motility-Indole-Urease) test, Citrate utilization test, Oxidase test, Catalase test, TSI agar test were carried out. The biochemical tests were triplicated for perfection and accuracy of the results.

2.4.1) Biochemical Identification

All the biochemical tests were performed with specific standard isolates developed in specific media according to the standard methods described in Microbiology Laboratory Manual [6]. All the bacterial cultures (both clinical and environmental) were grown on nutrient agar plates in the incubator at 37^0C before starting the process of any biochemical identification test.

MIU (Motility-Indole-Urease)

- The MIU media was prepared carefully into a screw capped test tube. The test tube acquired a deep orange color.
- A single colony of the bacterium to be tested was touched from a nutrient agar plate by using a needle. The colony was carefully stabbed straight into the test tube containing MIU.
- The test tube was incubated at 37^0C for 24 hours.
- The growth of the organism would spread throughout the test tube from downward to the upward of the test tube, if the organism is motile. The color of the media will turn to deep pink if the organism is positive for urease test.
- To confirm the indole test, five drops of Kovac's reagent were added following overnight incubation. Then the colors of the cultures were examined and the results were recorded. Formation of a rose red ring at the top indicates a positive result. A negative result can have a yellow or brown layer.
- The same procedure was carried out for the clinical and the environmental strains.

Citrate utilization test

- A small vial was taken into which a slant of the Simmon's citrate agar was prepared and allowed to solidify.
- A single colony of the bacterium to be tested was touched from a nutrient agar plate by using a needle and carefully streaked onto the slope of the citrate media.
- The vial was incubated at 37^0C for 24 hours. Over these 24 hours, if the organism had the ability to utilize citrate, it would change the color of the media from deep green Prussian blue. A negative result would keep the color unchanged.
- The same procedure was carried out for the clinical and the environmental strains.

Triple Sugar Iron (TSI) Test

- The TSI media was prepared into a screw capped test tube, and solidified to obtain a slant and butt at the length of the test tube.
- A single colony of the bacterium to be tested was touched from a nutrient agar plate by using a needle and carefully stabbed at the butt of the TSI (dextrose, lactose & sucrose) followed by slow streaking at the slant.
- The screw caps of the test tube were loosened and the tube was incubated at 35^0C for overnight.
- After the 18-24 hours of the incubation period, the test tube was examined to observe carbohydrate fermentation, Carbon dioxide (CO_2) and Hydrogen sulfide (H_2S) gas production.
- If the organism is able to ferment all the three sugars, then the butt would turn into yellow color indicating the production of acid and the subsequent decrease in the pH of the media, whereas a red color in the slant and butt indicated that the organism being tested is a non-fermenter and the media remains alkaline.
- Presence of bubbles, splitting and cracking of the medium is the indication of CO_2 gas production.
- A black precipitation in the butt of the tube is the indication of H_2S production.
- The same procedure was carried out for the clinical and the environmental strains.

Catalase test

- A number of autoclaved glass slides were taken, and a drop of the catalase reagent (Hydrogen peroxide) was placed on each of the glass slides.
- The glass slides were labeled according to the sample being tested.
- A colony for each of the bacteria to be tested was taken from a nutrient agar plate, and later placed onto the reagent drops on each of the glass slides.
- An immediate bubble formation indicated a positive result.
- The same procedure was carried out for the clinical and the environmental strains

Oxidase test

- A number of filter papers were taken, and two drops of oxidase reagent (p-Aminodimethylaniline oxalate) were added onto the filter papers (Whatman, 1MM).
- The filter papers were labeled according to the sample being tested.
- A loopful of each bacterium to be tested (the clinical and environmental strains) were taken from nutrient agar plate and streaked onto the filter paper (Whatman, 1MM).
- A positive reaction would turn the paper from violet to purple within 1 to 30 seconds. Delayed reactions should be ignored as that might give false positive result.

Methyl red (MR) test

- 6 ml of dextrose phosphate (MR-VP) broth was prepared in almost six test tubes.
- 3 ml from each of the test tubes were transferred to another six different empty test tubes.
- All the twelve test tubes were labeled according to the sample being tested and the test being conducted.
- All the bacteria samples to be tested were inoculated into 3 ml dextrose phosphate broth (MR-VP broth) which contained dextrose and a phosphate buffer and incubated at 37^0C for 24 hours.
- After the incubation period was over, five drops of the Methyl red reagent was added into the six test tubes (labeled as MR test) to check the pH of the medium. Development of a red color would indicate a positive result, whereas a yellow color would indicate a negative result.
- The same procedure was carried out for the clinical and the environmental strains

Voges–Proskaur test

- All the bacteria samples to be tested were inoculated into 3 ml dextrose phosphate broth (MR-VP broth) which contained dextrose and a phosphate buffer and incubated at 37^0C for 24 hours.
- After the incubation period was over, 10 drops of Barritt's reagent A was added to each of the test tubes and the cultures were shaken. Immediately, 10 drops of Barritt's reagent B was added and the cultures were shaken again.
- After 15 minutes, the colors of the cultures were examined and the results were recorded. Appearance of a red color was taken as a positive result.
- The same procedure was carried out for the clinical and the environmental strains.

2.4.2) Gram staining

- A colony of the bacterium to be stained was touched with a burnt loop and placed on a sterile glass slide.
- A drop of physiological saline was taken by the loop and then the bacterial colony was smeared on the glass slide with the drop of saline. The glass slide was heat fixed and the smear was allowed to dry.
- A drop of crystal violet was added to the smear and after one minute, the crystal violet was carefully washed off the glass slide.
- A drop of grams iodine was also added and then after one minute the grams iodine was carefully washed off the slide.
- 70% ethanol was added and washed off after 45 seconds.
- Saffronin was added and after 40 seconds it was washed off the glass slide.
- The slide was allowed to dry off completely, after which it was observed under the microscope.

2.4.3) Preparation of Stock Sample

Long term preservation

The media Trypticase Soy broth was prepared in a sterile cryovial. For long-term preservation, 500µl of bacterial culture grown in Trypticase Soy Broth at 37^0C for 6 hours. After the incubation period, 500µl of sterile glycerol was added to the broth culture and the cryovial was stored at -20^0C.

Short term preservation

Approximately 3ml of T1N1 media was prepared into sterile vials. Colonies from the bacteria samples to be preserved were touched by a needle from nutrient agar plates and stabbed onto the butt of the vials. Then the vials were incubated at 37^0C (SAARC) for 6 hours. After the incubation period was over, 200µl of paraffin oil was added into the surface of the medium contained in each of the vials. All the vials were carefully labeled and stored at room temperature.

2.4.4) Provided Antibiotic Discs

In this study, the potency of twelve different commercially available antibiotics was determined. They are listed below in the table:

Table 5: Provided Antibiotic Discs

Antibiotic	Disc identification number
Kanamycin	(k 30)
Erythromycin	(E 15)
Sulfamethoxazole / Trimethoprim	(SXT 25)
Pefloxacin	(PEF 5)
Streptomycin	(S 10)
Nalidixic Acid	(NA 30)
Cefoxitin	(FOX 30)
Gentamicin	(CN 10)
Nitrofurantoin	(F 300)
Oxacillin	(OX 1)
Chloramphenicol	(C 30)
Ciprofloxacin	(CIP 5)

2.5.1) Preparation of McFarland Solution

In all the microbiology laboratories, whenever antimicrobial susceptibility studies are carried out, one of the important techniques performed is the McFarland Turbidity Standard. McFarland standards are usually used as a reference in order to calibrate the turbidity of both the test and control microorganisms. One of the importances of this technique is that it allows the tests to be carried out with a defined concentration of the test and control microorganisms and avoids the possibilities of working with a high amount of the bacterial culture. Therefore it ensures that the number of bacteria is within a given range. This also averts the possibility of any sort of errors in the results, as too much concentrated or too much diluted bacterial suspension can lead to inaccurate outcomes about the potency of the antimicrobial agents against the test organisms.

- 97% H_2SO4 was diluted to a concentration of 1%
- $BaCl_2.2H_2O$ was diluted to a concentration of 1.175%
- To make 5 ml McFarland solution, 4975µl H_2SO_4 was mixed with 25µl $BaCl_2.2H_2O$.

2.5.2) Preparation of inoculums

- Significant amount of physiological saline was prepared in several test tubes. The test tubes were labeled carefully according to each of the samples for which the inoculum would be prepared.
- Using a burnt inoculating loop, one or two colonies of each of the bacterium to be tested were picked up from sub cultured nutrient agar plates.
- The colonies were later suspended into the test tubes containing the saline. The colony for each sample was suspended into the test tube labeled with the same sample.
- All the test tubes were vortexed properly so that the suspension becomes homogenous.
- The same procedure was carried out for the clinical and the environmental strain.

2.5.3) Comparison with McFarland solution

- Using the Colorimeter (Labtronics; ISO 9001: 2008 Certified) provided in the Microbiology and Biotechnology laboratory, the OD (optical density) of the McFarland solution was measured to be 11.
- The Colorimeter was also used to measure the OD of all the inoculums that was prepared. This was done to balance out the OD of these inoculums in the same range as the OD of the McFarland solution.
- The inoculums having the same OD to that of the McFarland solution was taken. The inoculums having a higher OD were diluted to bring the OD back into the reference range.
- After the OD of all the suspensions were in the same range as the McFarland solution, the inoculums were ready to be inoculated as pure lawn on MHA (Muller-Hinton Agar) plates.

2.5.4) Inoculation of the MHA plates

- Six different MHA (Muller-Hinton Agar) plates were prepared. Three of the plates were labeled for the clinical sample and three of the plates were labeled for the environmental sample.
- An autoclaved swab was dipped into the suspension and rotated so that the cotton is completely wet with the suspension of the organism. The test tube containing the suspension was vortexed before dipping the cotton swab. The swab was not dipped wet.
- The swab was later streaked at least four to six times onto the dried surface of the MHA plate (labeled as same as the suspension) to make a pure lawn over the entire agar surface, ensuring the contact of the cotton of the swab with all the corners of the plate. The agar plate was being rotated 90 degrees each time it was being streaked, in order to ensure the even distribution of the inoculum. The plates were allowed to dry out for three minutes approximately.

2.5.5) Placement of the Antibiotic Discs

- A sterile forceps was used to insert the antibiotic discs. Four different sterile antibiotic discs were placed on the surface of each of the inoculated MHA plates. All the different twelve antibiotic discs were placed in the same manner.
- After the discs were inserted, each of the discs was slightly pressed with the forceps so that it sticks properly to the agar surface.
- Inserting the discs close to the edge of the plates was avoided as the zones will not be fully round and that can be difficult to measure and lead to inaccuracy of the test.
- Once all the discs were properly placed, the MHA plates were inverted and incubated at 37^0C for 24 hours.
- The same procedure was carried out for the clinical and the environmental strains

2.5.6) Measuring zone size

- After the incubation period was over, the MHA plates were observed to identify the zone of inhibition for each of the antibiotics.
- The zones for each antibiotic were measured carefully, to the nearest millimeter using a ruler.
- All the measurements were made with the unaided eye while viewing the back of the Petri dish. The zone size was recorded on the recording sheet.

2.6 Protein profiling

The environmental strain was sub cultured into a fresh nutrient agar plate before conducting the whole protein profiling procedure. It was done 24 hours before protein extraction. The nutrient agar plate was incubated at 37^0C for 24 hours.

2.6.1 Protein Extraction

- For each of the both clinical and environmental strain, 5ml of autoclaved Luria Bertani broth was prepared in two different test tubes.
- The test tubes were carefully labeled according to each of the samples.
- Both the strains were inoculated into each of the test tubes using a burnt inoculating loop. The test tubes were incubated at 37^0C for 24 hours.
- After the incubation period was over, the cultures were taken into an eppendorf of 1ml after vortexing.
- Then the cultures were centrifuged at 14,000 rpm for approximately 5minutes. The supernatant was discarded. The pellet was suspended at the bottom of the tube.
- Using a micropipette 4x SDS dye of 20µl was added in each eppendorf.
- The butt of the tube was tapped vigorously to dissolve the pellet and then the eppendorf were suspended at the water bath to boil at 100^0C for 3 minutes.

- After the boiling, the eppendorf was stored at -20^{0}C refrigerator to preserve the protein.

2.6.2 Preparing SDS-PAGE gels

Step (1):

- All the plates and combs were properly cleaned. One short plate was needed for each gel.
- One spacer plate and one comb were also required.
- All the plates were sprayed with a small amount of 70% ethanol, and later the plates were wiped dry with Kim wipes.
- The combs were thoroughly washed with tap water.
- It was essential to remove all the dust, small particles, and any leftover polyacrylamide properly.

Step (2):

- The plates were set properly on the rack.
- The short plate and the spacer plate were slide into the holder by layering each of the plates with the spacers in between.
- The edges at the bottom of the two plates were flushed to avoid any sort of leakage.
- The plates were locked in and the holder was placed on the rack with the bottom edges of the plates pushed into the foam/ rubber pad to make a water-tight seal.
- A small volume of water was pipetted in between the plates to ensure that there was no leakage.
- The blot was dried with filter paper.

Step (3):

- The separating gel was carefully poured after calculating the total volume properly.
- While pouring bubbles were avoided, which otherwise could stop polymerization.
- After adding each component, the solution was mixed by gently swirling it.
- Once TEMED was added, the gel would begin to polymerize. This step was conducted very carefully.
- The gel was pipetted and mixed between the plates in a careful manner so that there was enough space at the top for the stacking gel and the comb.
- Water was carefully poured over the gel solution.
- After 10-15 minutes, once the gel was polymerized, the top of the gel was washed of with water.
- With the use of a filter paper excess water was carefully blotted, and it was ensured that the gel was not disturbed.

	7.5% gel	10% gel	12.5% gel	15% gel	18% gel
ddH_2O	2.81ml	2.5ml	2.19ml	1.88ml	1.5ml
40% acrylamide/bis stock	0.94ml	1.25ml	1.56ml	1.88ml	2.25ml
1.5M Tris, pH 8.8	1.25ml	1.25ml	1.25ml	1.25ml	1.25ml
10% ammonium persulfate	50µl	50µl	50µl	50µl	50µl
TEMED	5µl	5µl	5µl	5µl	5µl
Total volume	~ 5ml	~ 5ml	~ 5ml	~ 5ml	~ 5ml

Step (4):

- The stacking gel was poured carefully.
- After adding each component, the solution was mixed by gently swirling it.
- The gel was mixed by pipetting between the plates, up to just below the edge of the short plate.
- The comb was placed carefully.
- After the gel was polymerized, the comb was removed slowly.
- The wells were carefully washed.

ddH_20	3.13ml
40%acrylamide/bis stock	0.62ml
1.5M Tris, pH 6.8	1.25ml
10% ammonium persulfate	50µl
TEMED	5µl
Total volume	~5ml

Step (5):

- The comb was returned to storage and the gel was sandwiched between two wet pieces of paper towels.
- The gel was stored at 4°C for two days.

2.6.3 SDS-PAGE

- <u>Resolving (running) gel is prepared and added-</u>
    a) The resolving gel is prepared up to 1.5mm in length
    b) The plates and combs were cleaned.
    c) The plates were set up on the rack and fixed with the casting stand.
    d) All the ingredients were added together, except APS (ammonium persulfate) and TEMED (, N,N,N',N'-tetramethylethylenediamine) because both of these solidify very fast and polymerizes rapidly.
    e) 100% ethanol was also added so that no bubble is present in the gel.

f) The gel was marked with a comb before adding the resolving gel.
g) The ethanol does not solidify as the filter paper is used to soak it out as it stays at the top layer.
h) The resolving gel would take approximately 30 minutes to polymerize.

- Stacking gel is prepared and added –
  a) 5ml of 10% of stacking gel was prepared up to 1.5mm in length.
  b) Ethanol was poured off at first & the watery inner plate surface was dried with a piece of Whatmann paper.
  c) 1ml of 0.625 Tris-HCL was added.
  d) APS/TEMED was added.
  e) The gel was marked with comb to make wells.
  f) It was then mixed and the polymerized resolving gel was poured on top.
  g) The comb was inserted carefully straight into the gel.
  h) To fully the sides of the comb, a little more stacking gel was poured.
  i) Any bubbles were removed from underneath the comb. The bubbles were allowed to float up by slightly moving the comb sideways.
  j) The stacking gel polymerized within 30 minutes.

- Load the gel-
  a) The electrophoresis buffer (Tris glycerine) was poured into the upper & lower chambers.
  b) The wells were flushed with a syringe just before loading to get rid of any un-polymerized polyacrylamide that may sweep in.
  a) The samples were loaded and the gel was run at 150 volts for 1 hour.
  b) The gel run was ensured seeing bubble forming.
  c) The gel was placed in a plastic container and covered with isopropanol fixing solution. It was shaken at room temperature. For 0.75 mm thick gels (10-15 minutes shaking) and for 1.5mm thick gels (30-60 minutes shaking).
- Staining & de-staining-
  a) The SDS-PAGE gel was removed from glass and rinsed once in ddH2O in a suitable container with a lid.
  b) The fixing solution was poured off and covered with Coomasie blue staining solution. It was shaken at 55ºC water bath for 1.5 hours.
  c) The staining solution was poured off and the gel was washed with 10% acetic acid for de-staining.
  d) Fresh De-staining solution was added to cover the gel by 3/4 inch (~ 2 cm) and was incubated at water bath for 1 hour.
  e) The bands were observed and measured with a ruler to determine the distance travelled by the dye front and different protein bands.

Chapter 3: Results

Results

3.1) Conformation of the clinical strain

a) The clinical strain of the bacterial species i.e. *Salmonella typhi*, obtained from ICDDR,B (International Center for Diarrheal Disease Research, Bangladesh) were streaked in the respective selective media (Table 2) in order to determine and confirm the cultural properties of the organisms. Selective media are specialized ones which support the growth of a particular desirable organism, while inhibits the growth of the other organisms. These media contain antimicrobials, dyes, or alcohol to inhibit the growth of the organisms that are not targeted for study.

Table 6: Cultural characteristics of clinical strains on respective selective

Isolates/ Organism	Medium	size	Margin	Elevation	Form	Pigment	Consistency
S. typhi	XLD	Moderate (2-3) mm	Entire	Raised	circular	Red colonies with black center	smooth

Figure 1: Cultural characteristic of clinical strain on respective selective media: *Salmonella typhi* in Xylose Lysine Deoxycholate agar

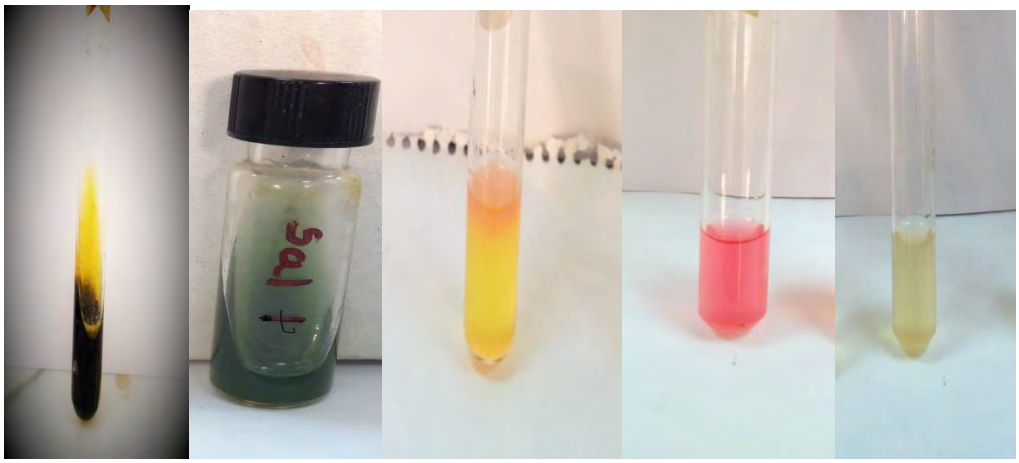
The colonies isolated from Figure 1 were cultured into fresh nutrient agar plates. The cultures were later subjected to biochemical tests. The isolates showed patterns of biochemical reactions that are typical for each strain of bacteria according to Microbiology Laboratory Manual [6].

The table below (Table 7) represents the results for the biochemical tests:

Table 7: Standard results of biochemical tests of target isolates

Isolate/Organism	MIU Test	Methyl red test	Voges Proskaur test	Citrate utilization Test	Catalase Test	Oxidase Test	TSI Fermentation Test			
							slant	Butt	CO_2	H_2S
Salmonella typhi	-	+	-	-	+	-	**K**	**A**	-	+

KEY: A= acidic condition, K= alkaline condition, + = positive, - = negative, AG= both acid & gas production.



Left to right (TSI, Citrate, MIU, Methyl red, Vogues-Proskaur)

Figure 2: Biochemical tests for *S. typhi*

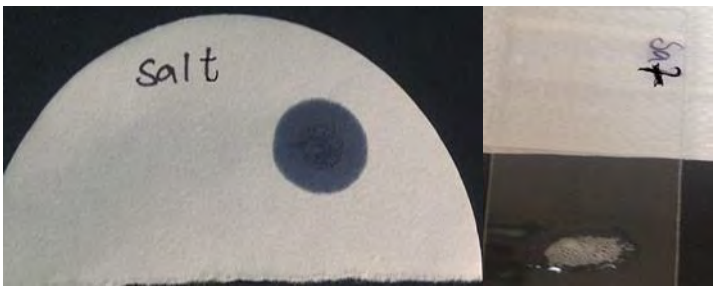


Fig 3: Oxidase (left) & Catalase (right) test for *Sal typhi*

3.2) Confirmation of the environmental strains by biochemical tests.

All the aimed and presumptive environmental strains of *Salmonella typhi* are selected from specific media (Table 2) depending on their cultural characteristics (Table 8).

Table 8: Cultural characteristics of the environmental strains

Isolates/ Organisms	Medium	Size	Margin	Elevation	Form	Pigment	Consistency
Sal 1	XLD	Moderate (2-3) mm	Entire	Raised	Circular	Red colonies with black center	Smooth
Sal 2	XLD	Small (1-2) mm	Entire	Slightly raised	Circular	Black colonies	Creamy
Sal 3	XLD	Moderate (2-3) mm	Entire	Slightly raised	Convex	Red colonies with black center	Creamy
Sal 4	XLD	Moderate (2-3) mm	Entire	Raised	Convex	Red colonies with black center	Smooth
Sal 5	XLD	Moderate (2-3) mm	Entire	Raised	Circular	Red colonies with black center	Smooth

Multiple number of colonies (approximately eighteen) were isolated based on their standard cultural properties and later were subjected to biochemical tests for further confirmation.

Table 9: Biochemical test results of the environmental strains

Isolate/Organism	MU Test			Methyl red Test	Voges-Proskaur Test	Citrate utilization Test	Catalase Test	Oxidase Test	TSI test			
	Indole test	Motility Test	Urease Test						Slant	Butt	CO_2	H_2S
Sal 1	-	+	-	+	-	-	+	-	**K**	**A**	-	+
Sal 2	-	+	-	+	-	-	+	-	**A**	A	+	-
Sal 3	+	+	-	+	-	-	+	-	**K**	**A**	-	+
Sal 4	+	+	-	+	-	-	+	-	**K**	**A**	+	-
Sal 5	+	+	+	+	-	-	+	-	**K**	**A**	-	+

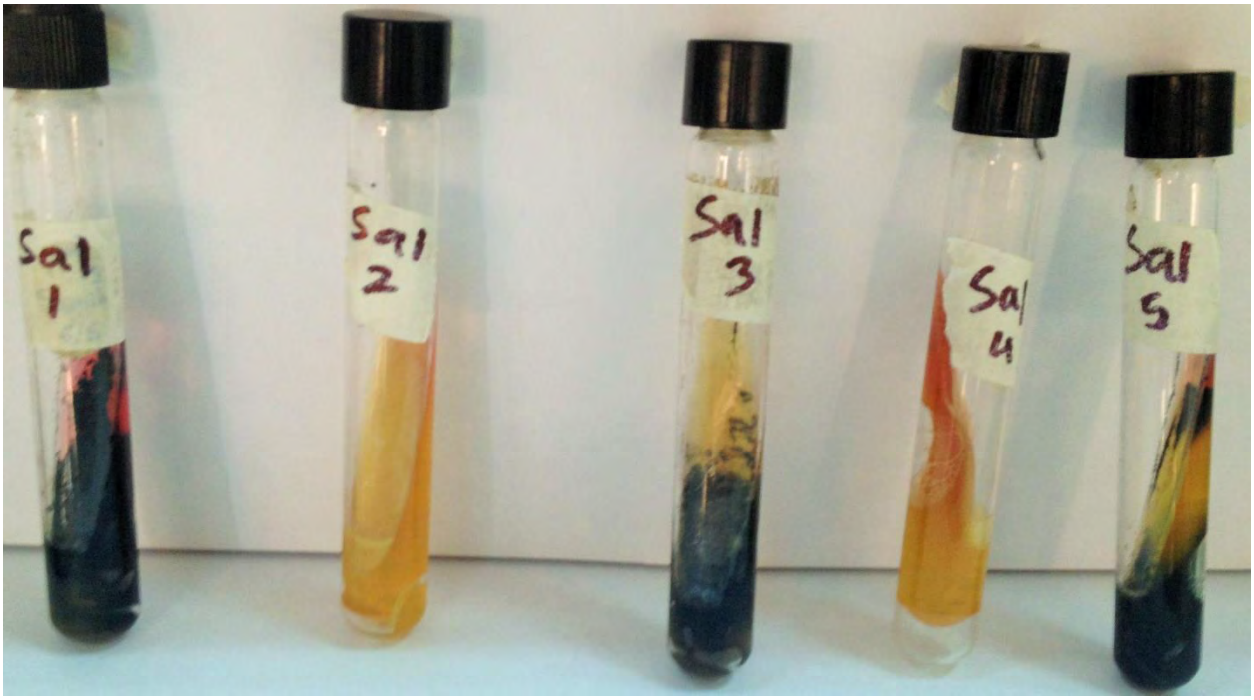


Fig 4: TSI test for five environmental strains of *Salmonella spp*.

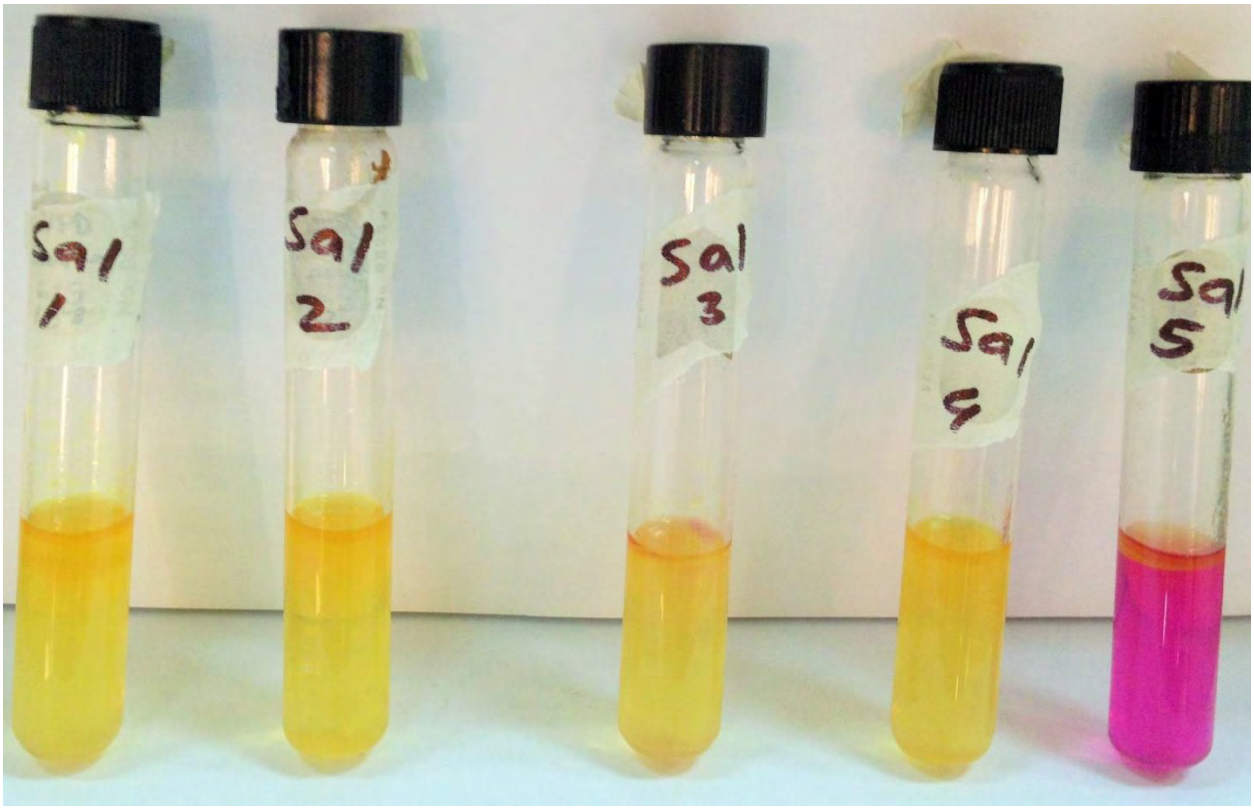


Fig 5: MIU test for five environmental strains of *Salmonella spp*

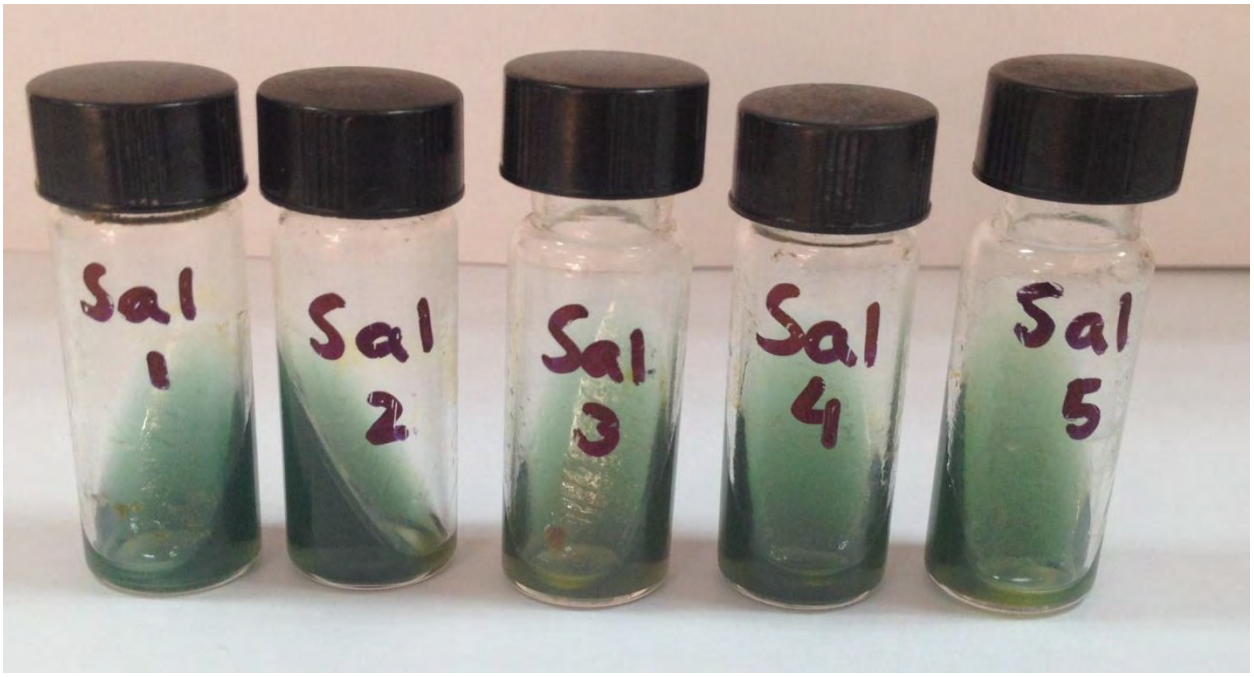


Fig 6: Citrate utilization test for five environmental strains of *Salmonella spp*

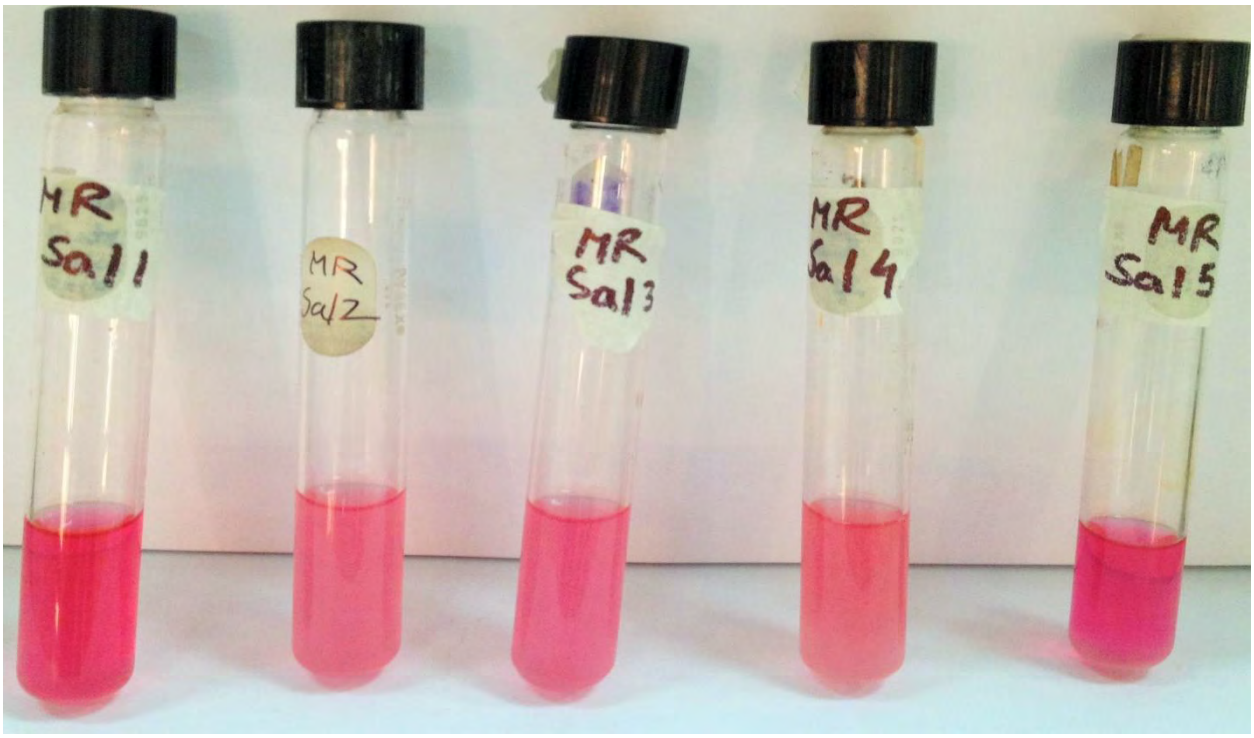


Fig 7: Methyl red test for five environmental strains of *Salmonella spp*

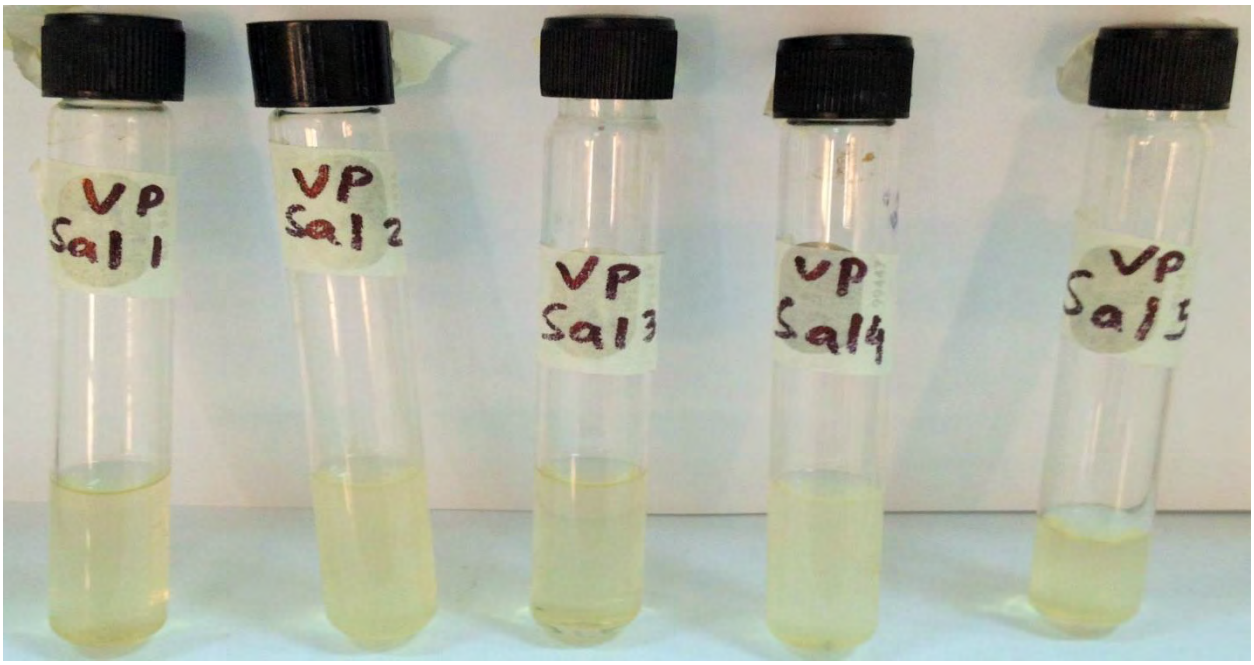


Fig 8: Voges-Proskaur test for five environmental strains of *Salmonella spp*

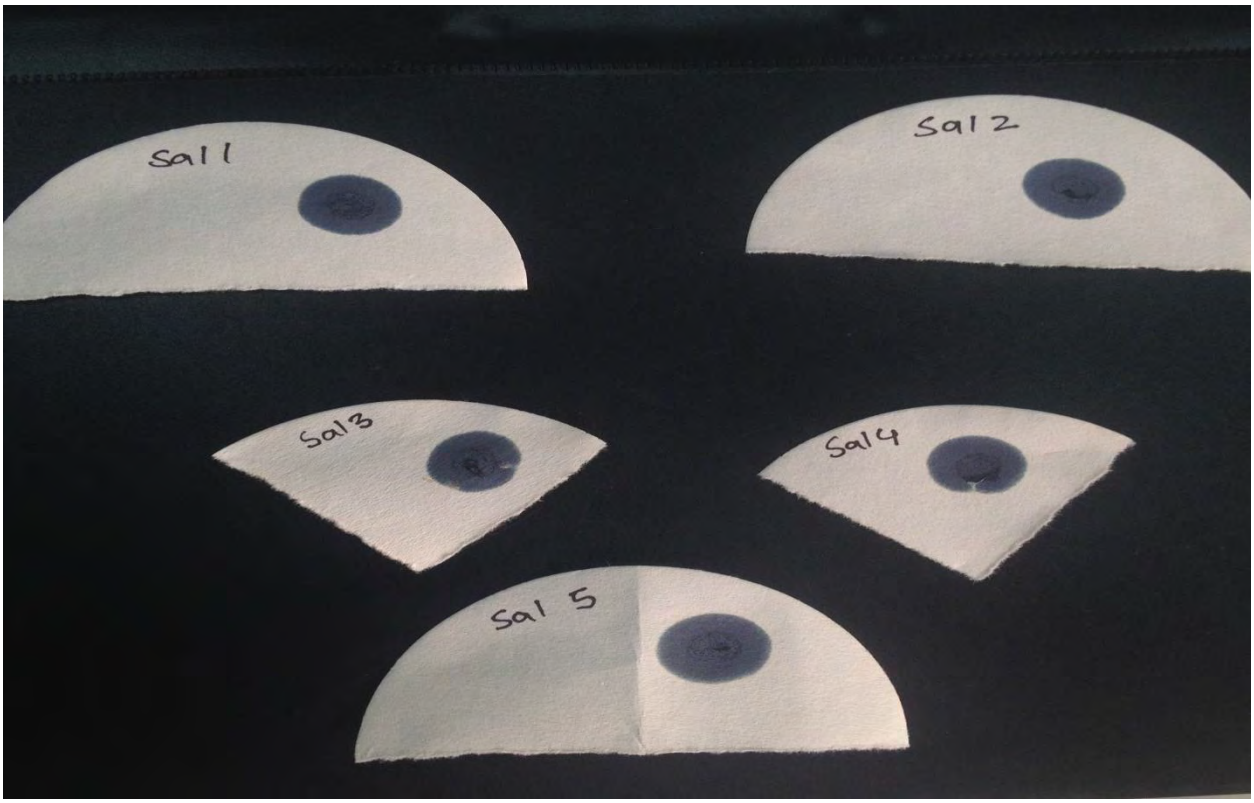


Fig 9: Oxidase test for five environmental strains of *Salmonella spp.*

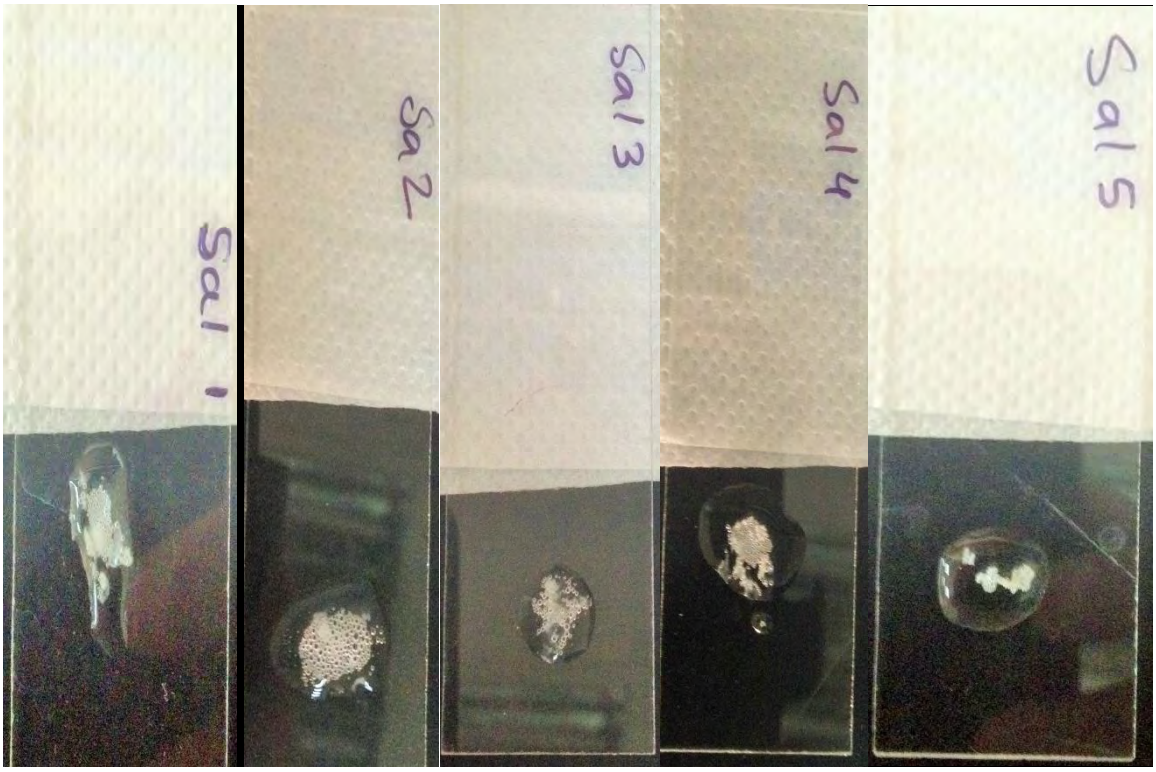


Fig 10: Catalase test for five environmental strains of *Salmonella spp*

3.3) Morphology confirmation of both the Clinical strain and Environmental strains via Gram staining.

After deciding that the study would be conducted the clinical strain (*S.typhi*), the morphology was checked for that particular strain and all the environmental strains that gave coinciding biochemical results for *Salmonella typhi.*

Table 10: Morphological properties of the clinical and environmental strains

Isolate/organism	Shape under the microscope	Color
Salmonella typhi	Rods	red
Sal 1	Rods	Red
Sal 2	Rods	red
Sal 3	Rods	red
Sal 4	Small rods	red
Sal 5	Rods	red

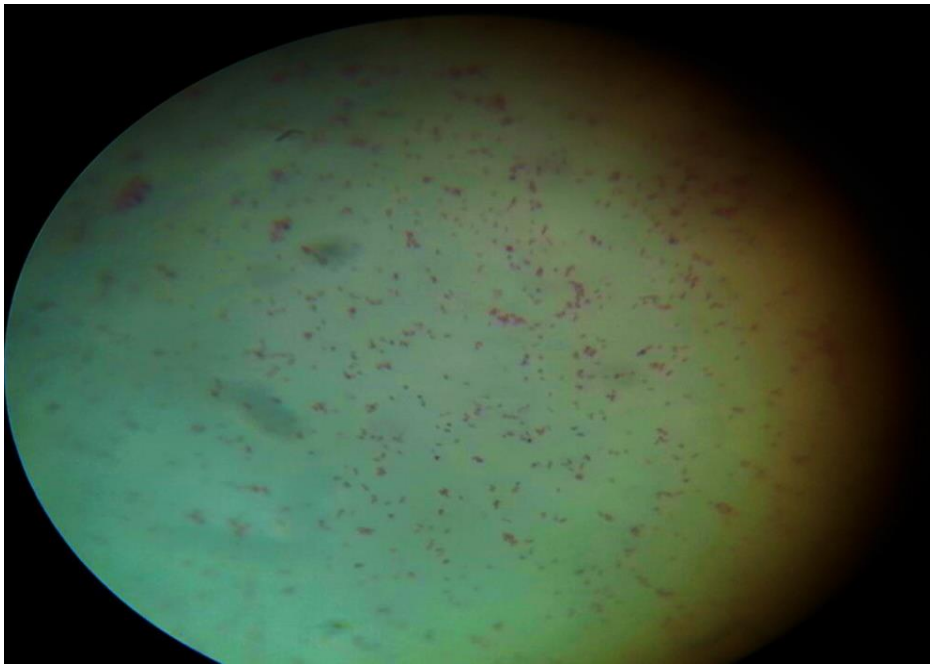


Fig 11: Gram –ve *S.typhi* under the microscope

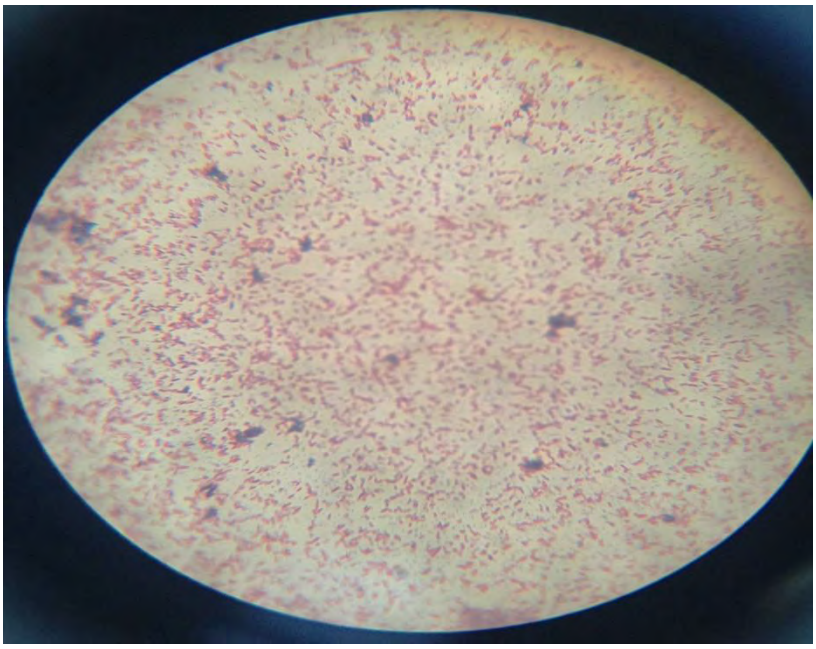


Fig 12: Sal1 under the microscope

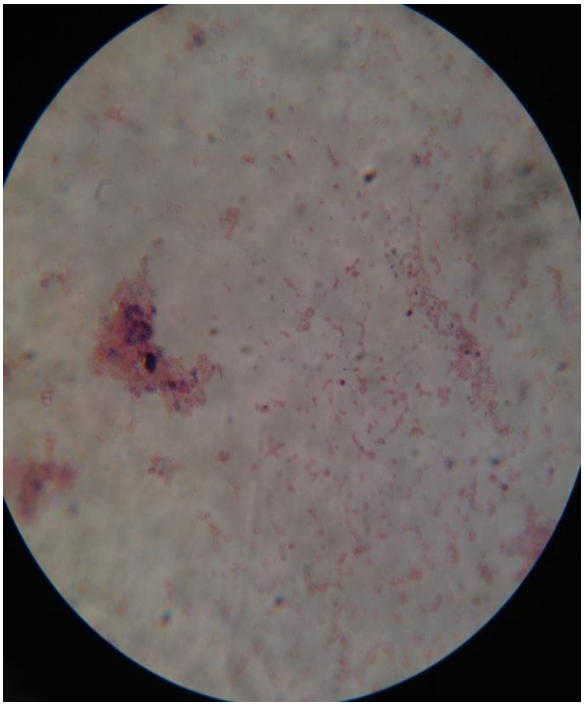


Fig 13: Sal2 under the microscope

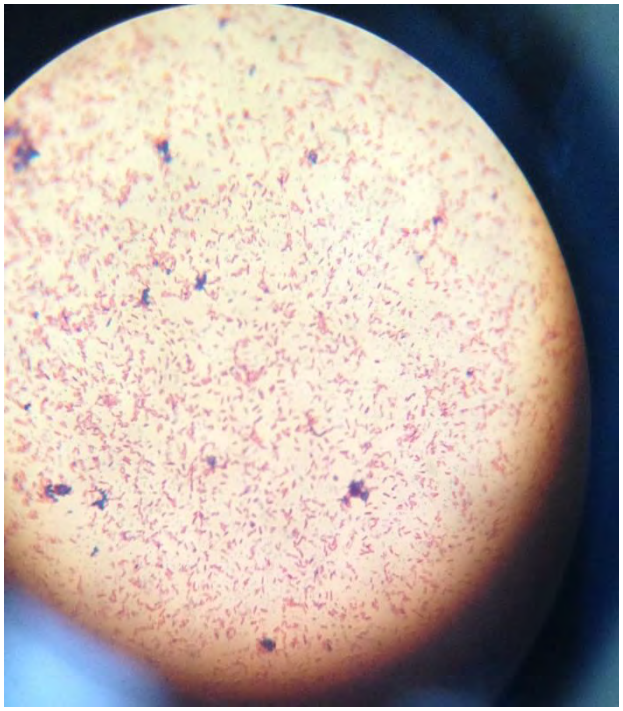


Fig 14: Sal3 under the microscope

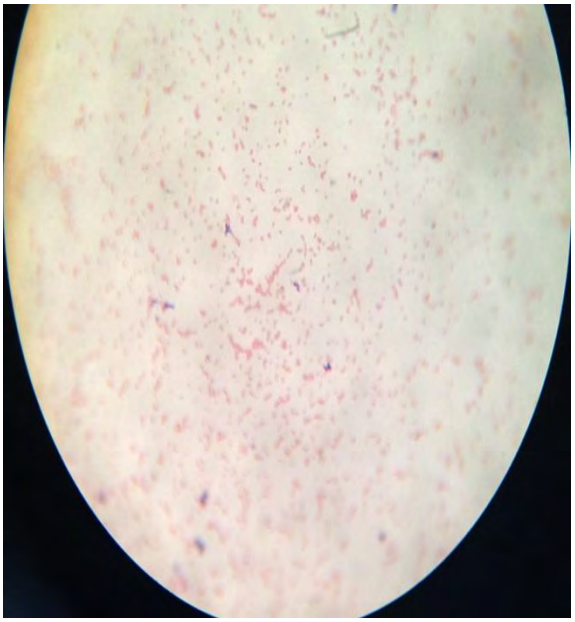


Fig 15: Sal4 under the microscope

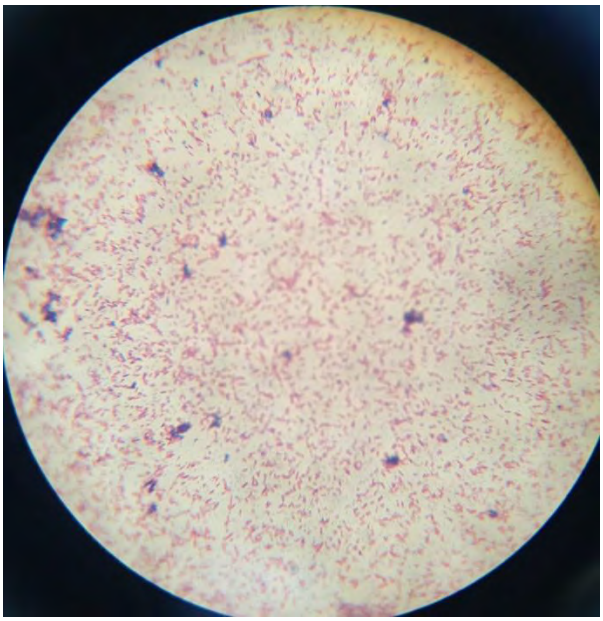


Fig 16: Sal5 under the microscope

After conducting all the biochemical and morphological tests, Sal1 was chosen to be the strain that gave very similar and almost identical results when compared with the standard results. The study from here on has been conducted with the strain Sal1 (Environmental) and *Salmonella typhi* (Clinical). The antibacterial resistance pattern for both the strains were tested, observed and compared.

3.4) Selective antimicrobial activity test by means of antibiogram method

All the four strains which were confirmed to carry out the test further was later subjected to the standard disc diffusion test with the available antibiotics (Table 5). The zone diameter of inhibition elucidates and interprets the resistance and sensitivity of the organisms to the respective antibiotics. All the clinical strains showcased significant resistance to all three OX1, NA30 &E15. *Salmonella typhi* was also fully resistant to FOX30. *Salmonella typhi* was found to be most susceptible to K30 with a zone of 48mm. In case of the environmental strain, it showed full resistance to OX1. The environmental strain of *Salmonella* (Sal1) showed most susceptibility FOX30 with a zone of 27mm.

.

Table 11: Antibiotic susceptibility test results for clinical strains and the chosen environmental strains

Antibiotic disc	*Salmonella*	
	Clinical	Environmental
PEF 5	20 mm [S]	26 mm [S]
CIP 5	43 mm [S]	13 mm [I]
C 30	14 mm [I]	19 mm [I]
K 30	48 mm [S]	20 mm [S]
SXT 25	14 mm [I]	24 mm [S]
OX 1	0 mm [R]	0 mm [R]
NA 30	0 mm [R]	21 mm [S]
E 15	0 mm [R]	13 mm [I]
S 10	15 mm [I]	16 mm [I]
CN 10	37 mm [S]	8 mm [I]
F 300	27 mm [S]	19 mm [I]
FOX 30/NIT300	0 mm [R]	27 mm [S]

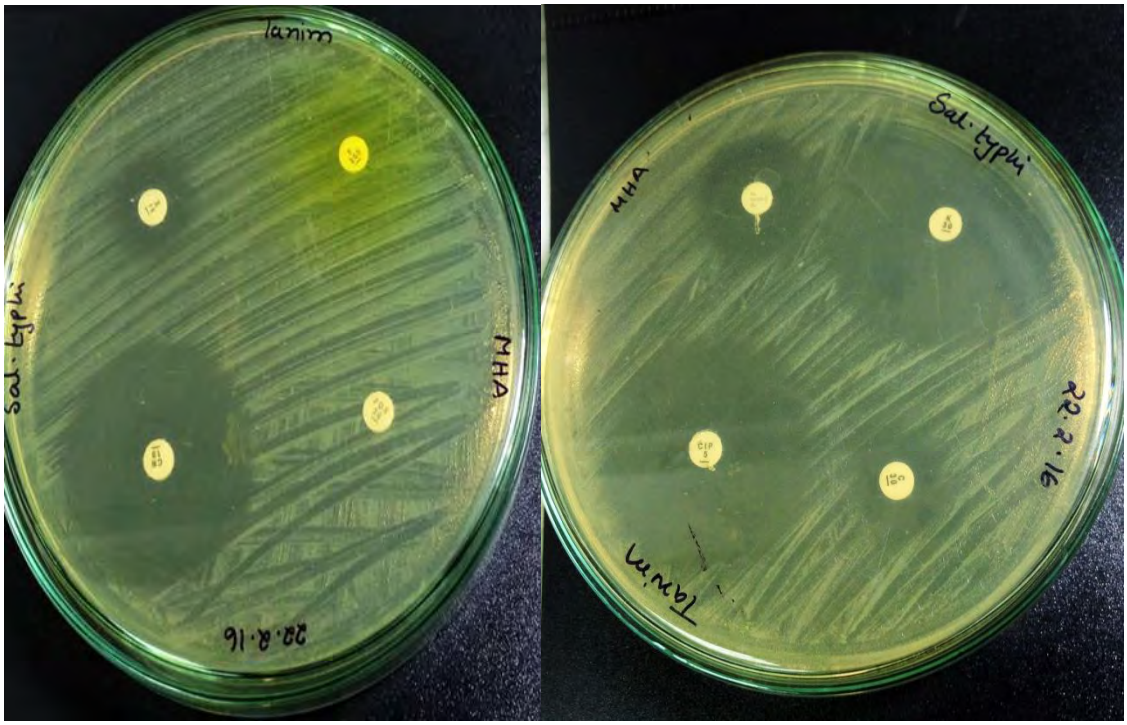


Figure 17: Effect of the applied antibiotics on clinical strain of *S. typhi*

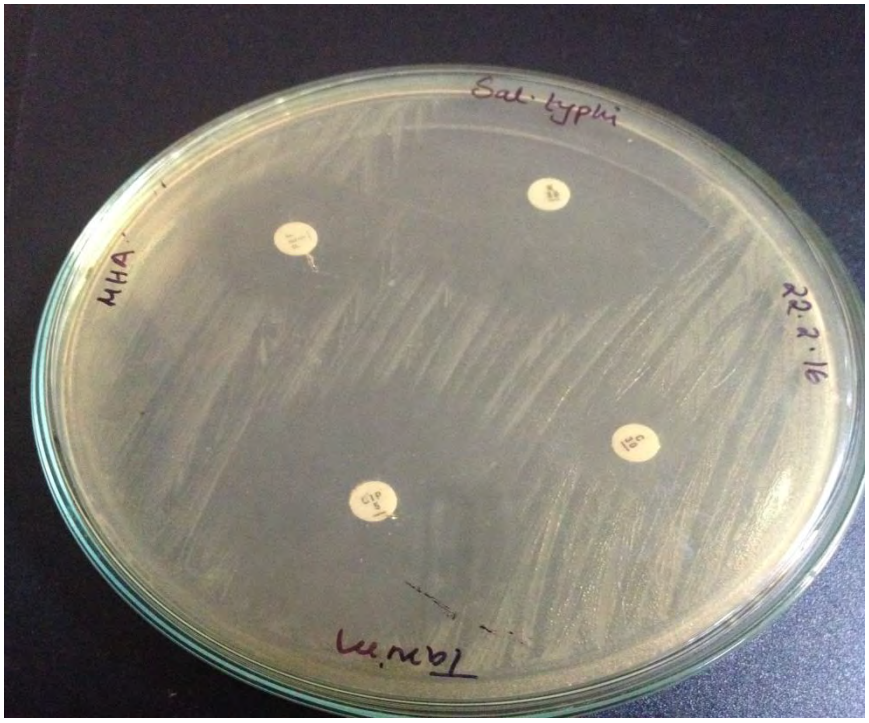


Figure 18: Effect of the applied antibiotics on clinical strain of *S. typhi*

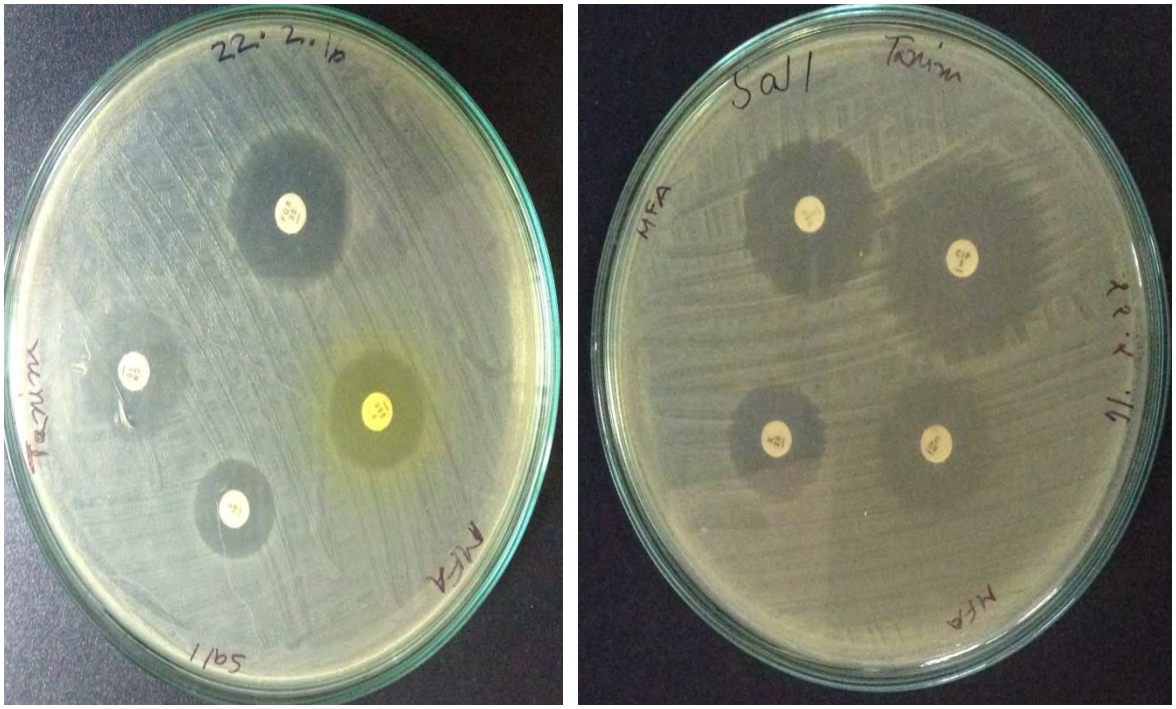


Figure 19: Effect of provided antibiotics on Sal1

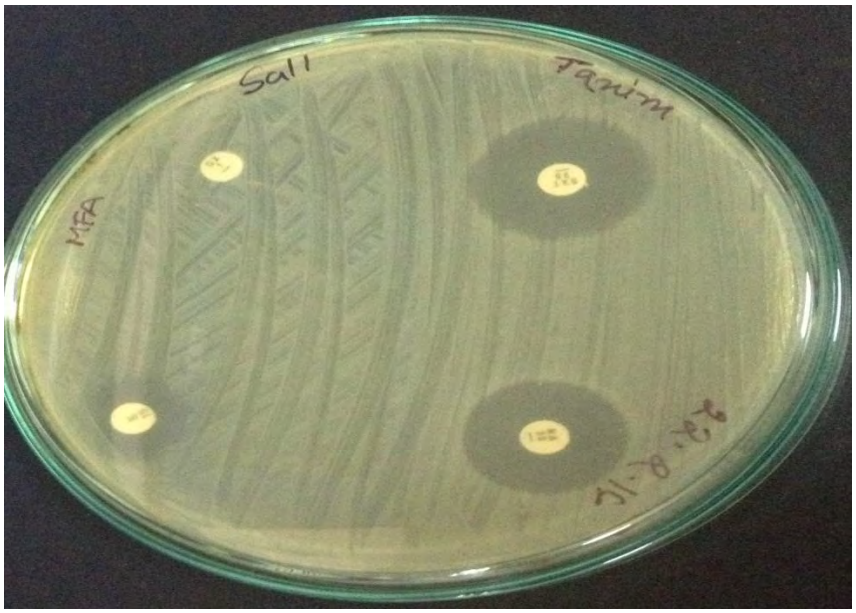


Figure 20: Effect of provided antibiotics on Sal1

3.5) Comparative analysis of antibiotic susceptibility profiles of clinical and environmental strains

The profile for the susceptibility of the clinical and environmental strain of *S.typhi spp* showed significant and distinguished pattern. It can be briefed out by stating that it's not necessary that a particular antibiotic will have the same effect on the environmental strain of a chosen organism as it has on its clinical strain. There are various factors and key changes that influence these significant variations, which will be understood and discussed only after the protein profiling of the strains are completed.

In case of *S.typhi spp*, the clinical strain showed full resistance against four of the antibiotic discs named as OX1, E15, FOX30 & NA30, whereas the environmental strain showed significant sensitivity against all these antibiotic discs except OX1. The only coinciding result for both the clinical and the environmental strain was that both showed a 0 mm zone against OX1.

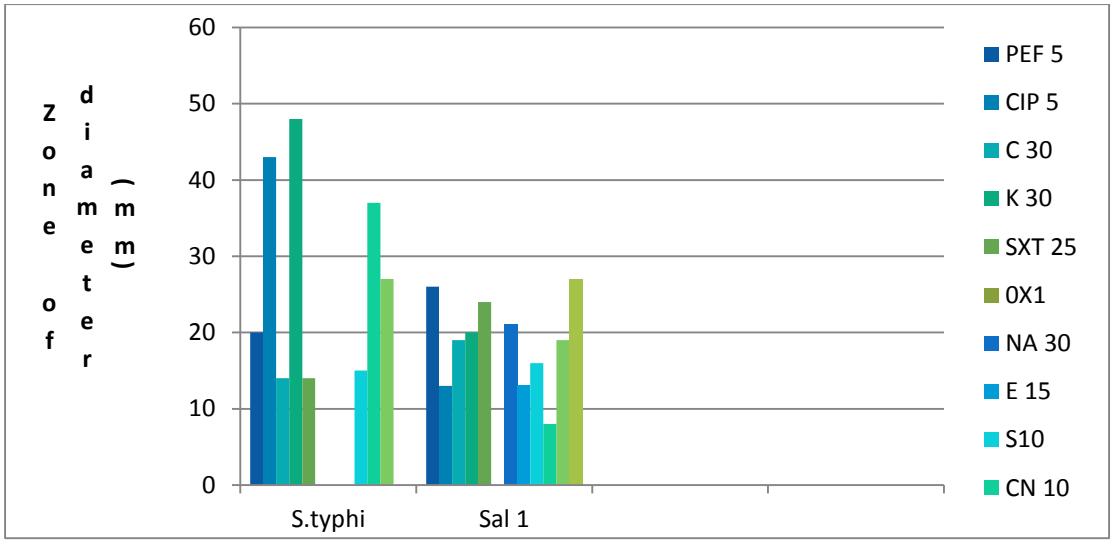


Figure 21: Comparative analysis of the effect of different antibiotics on clinical and environmental *Salmonella typhi* on a bar chart

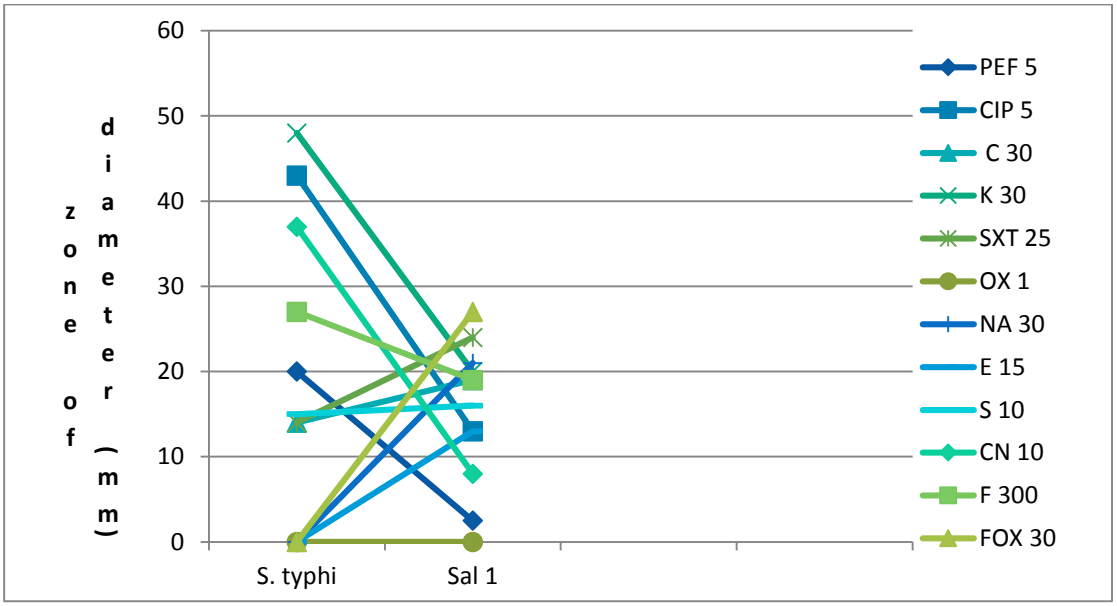


Figure 22: Comparative analysis of the effect of different antibiotics on clinical and environmental *Salmonella typhi* on a graph

3.6) Qualitative analysis of SDS page result

In order to determine the molecular weight of unknown proteins, SDS PAGE is one of the most perfect and feasible techniques that should be applied. When SDS PAGE is applied with the unknown proteins, the proteins migrate through the polyacrylamide gel in which the migration rate is inversely proportional to the logarithm of the molecular weight (MW) of the respective proteins. To achieve a more reliable and a significant result, it is appropriate to carry out the process in different conditions that lead to a linear relationship between the log MW and the migration rate of the proteins within the known molecular weight range of the proteins.

The same gel was used to run all the proteins of all the strains simultaneously. This was done in order to acquire a more reliable and qualitative MW determination.

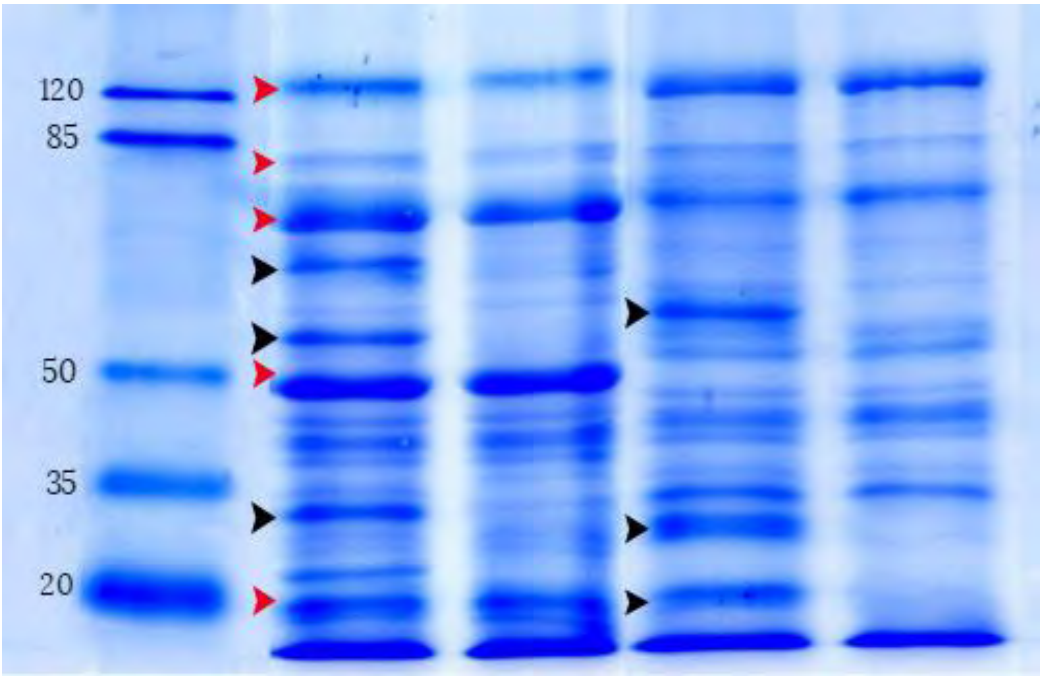


Figure 23: SDS page result analysis of *S. typhi* (clinical and environmental) whole cell protein (Red arrows indicate known proteins and black arrows indicate unknown proteins)

After the proteins were separated by SDS PAGE, the bands on the gel were observed and the relative migration distance (Rf) for the marker and the unknown proteins were determined. The relative mobility of the proteins is always related to their molecular mass.

The Rf is calculated using the following equation:

Rf = (migration distance of the protein) / (migration distance of the dye front)

Usually Rf is defined as the distance travelled by the protein divided by distance travelled by the ion front, but as the distance travelled by the ion front is difficult to locate and calculate, the distance travelled by the dye front is used as an alternative to calculate the value.

Table12: Relative migration distance of protein markers and respective log of MW

Molecular weights of Marker Proteins	Migration distance of the protein (mm)	Migration distance of the dye front (mm)	Rf	Log of MW
120	1	7.5	0.133	2.68
80	1.9	7.5	0.253	1.90
71	2.5	7.5	0.33	1.85
50	4.5	7.5	0.6	1.70
20	6.6	7.5	0.88	1.30

Using the values of the log MW and their Rf values for the marker proteins, a plot was constructed in order to determine the dependence of the migration rate of the proteins on their molecular weights. The plot obtained was not exactly linear, and therefore a best fit line was drawn in order to find out the weight of the unknown proteins. In case of extremely high MW proteins, the standard curve is usually a sigmoid curve as the sieving effect of the gel matrix is so large that the proteins find it very difficult to penetrate through this frictional resistance of the matrix. In case of very low MW proteins, the sieving effect is trivial and the proteins almost move freely through the gel matrix.

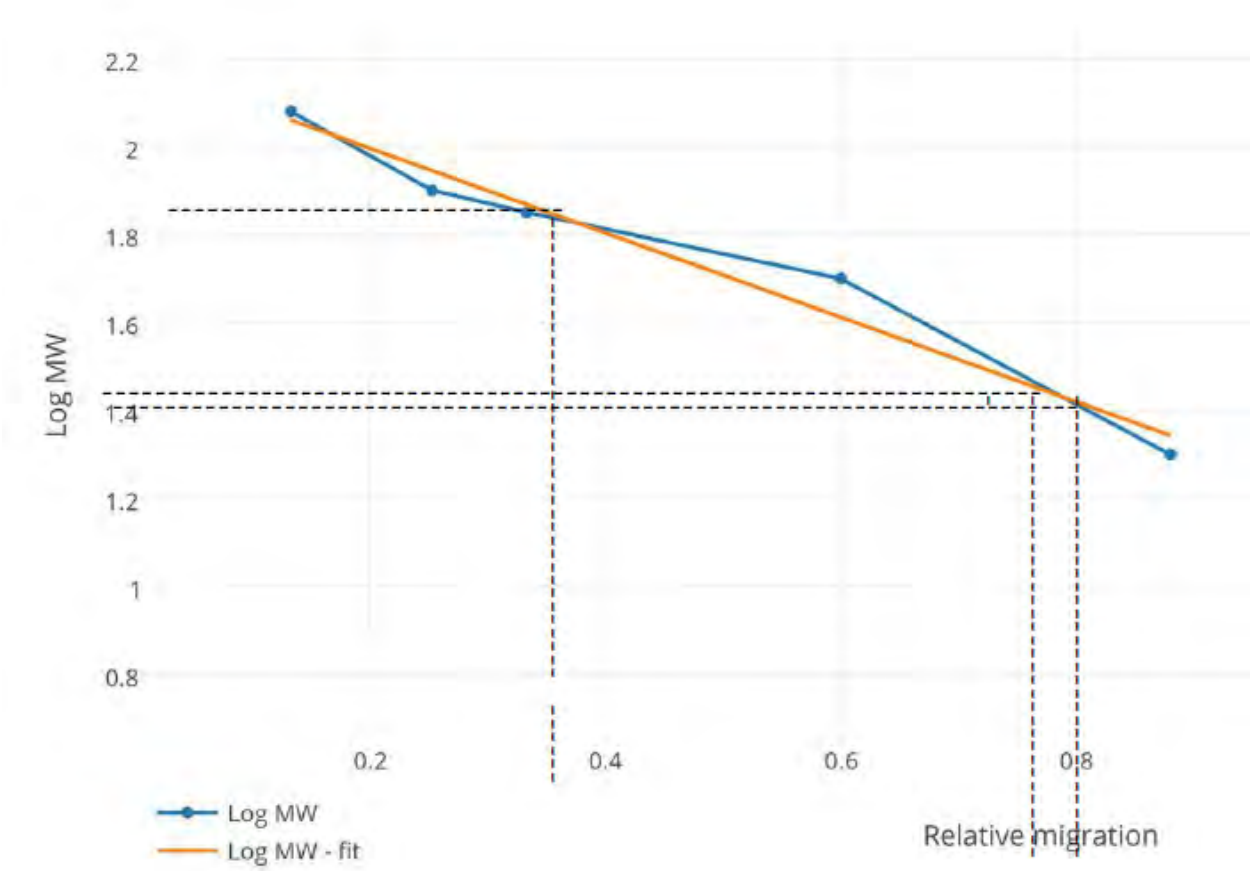


Fig 24: Plot of Log MW against Rf distance

The molecular weights of the unknown proteins were interpolated from the 'best fit' graph of Log MW i.e. it's the inverse log of the Log MW values. The Log MW of the unknown proteins bands corresponding to their relative migration are as follows:

Table 13: Interpolation of MW of unknown proteins

Organism	Migration distance (cm)	Rf	Log MW	Molecular weight (kDa)
S.typhi	3.5	0.37	1.83	67.61
	6.1	0.81	1.4	21.4
	6.7	0.89	1.33	25.1

Chapter 4: Discussion

Discussion

Antibiotic resistance spreading massively over the populations of the developing countries has been one of the vital concerns of the 20th century. The development of resistance against the most well effective and popular antibiotics is assumed to be a natural phenomenon now. When antibiotics fail to effectively kill the bacteria under therapeutic levels, it is understood that certainly this is an outcome of development of resistance by the bacteria, which can be acquired in a couple of ways. When the susceptible bacteria are inhibited or killed by a dosage of a particular antibiotic, it exerts a selective pressure for the endurance levels of the resistant strains of the same bacteria.

Research and studies have shown that both bacterial genetic makeup and human action can play key roles in encouraging the development of resistance against antibiotics. It is a known fact that antibiotics are the secondary products of bacteria themselves, and hence it is not amusing at all that bacteria which can produce such antibiotics use these products against other bacteria in a competitive habitat. This triggers natural selection in low levels leading to the development of antibiotic resistance. On the contrary, the usage of antibiotic has been exploited over the decades, and in such a manner that it can be stated as a kind of antibiotic abuse. With the digitalization and rapid pharmaceutical discoveries, humans have been more or less dependent on primary antibiotics for every kind of infection. In some countries, patients purchase antibiotic over the internet without a doctor's consent, and in some countries patients consume antibiotics unnecessarily to treat viral infections.

One of the most fascinating way in which bacteria can acquire resistance is through spontaneous mutations in their genetic makeup. It is believed that the mutations inactivate the potency of the antibiotic by either eliminating the target portion of the ell which the antibiotics tend to attack, or by making the bacteria release chemicals that can inactivate the antibiotic itself. In many cases bacteria are able to change seal the entry ports so that the antibiotic fails to enter the cell. In either of the way, such bacteria are naturally acquiring the resistance and hence induce the threat of the spread of resistance. Another way in which bacteria can acquire resistance is via conjugation method, where plasmids from resistant bacteria are transferred to sensitive bacteria, resulting in the development of resistance and hence the entire population slowly acquires the phenomenon. A single bacteria population can acquire resistance to multiple antibiotics in a single time. Horizontal gene transfer is known to be the ultimate mechanism via which bacteria can spread the resistance [1]. Human actions can also lead to the spread of bacterial resistance as some infections are contagious and can be passed on by coughing or any kind of physical contact.

The above study led us to some crucial conclusions about both the organisms studied here. The clinical isolates obtained from ICDDR,(International Center for Diarrheal Disease Research, Bangladesh) were first confirmed by various biochemical tests and gram staining. It was ensured that the strains remain viable and hence was maintained in nutrient agar media. The environmental strains of *Salmonella typhi* were isolated from different potential environmental sources like salad sample and raw fish water. These target strains were later on used to identify the antibiotic resistance pattern in comparison with the clinical strains.

In this study the clinical isolate of and *S.typhi* were found to be fully resistant to four different antibiotics namely Oxacillin, Nalidixic acid, Erythromycin and Cefoxitin. The significance of such a pattern can be thought to be due to several reasons, which could be complicated but yet justifiable. It is a known fact that *S.typhi* is an enteric organism and usually causes typhoid fever and diarrhea respectively amongst populations under a major outbreak. This can be elaborated with the assumption that as 80% of the population of Bangladesh rural, illiterate and un-urbanized, people are unaware about the causes and effects of a major outbreak of enteric diseases which are witnessed in many local and rural areas almost every year. The number of cases of diarrhea caused by *Salmonella spp* are increasing in finite numbers every year, and especially during seasonal floods are the predominant organism in both the flood and non-flood periods, whereas *Salmonella spp* has shown significant outbreaks during such seasons. Such outbreaks are difficult to be treated frequently and rapidly in order to decrease the percentage of fatality rates within the population. According to studies, almost 10 million children under 5 years die worldwide due to such infections. According to a recent study, multi-drug resistant typhoid fever is endemic in countries like India and Bangladesh and at least 21.7 million cases emerge every year [7]. The outbreaks have been only becoming common and thus the treatments and over usage of the common antibiotics as medication have become full-fledged especially in developing countries like Bangladesh. The over usage of such antibiotics which were once strong and evident to eradicate such infections, has led to the development of resistant strains against them, more and more people consuming these antibiotics has led to their popularity amongst the local markets, where people without much knowledge about the dosage and longevity of the antibiotics have consumed such antibiotics and might have also stopped the consumption prematurely leading to the development of more resistant strains amongst the population. For example, it was officially declared that how in 1948 Chloramphenicol was introduced by T.Woodward as an effective remedy for enteric fever caused by *S.typhi*, but during the 1970s, chloramphenicol multi-drug resistant strains of S.typhi strains with plasmid mediated genes were already isolated from many areas as an outcome of its over usage as 1st line drugs [7]. Situation in Bangladesh has worsened over the last 20 years, Ciprofloxacin is no more prescribed as an empirical drug to treat enteric infections since in 2000 the resistance against it was 8% and by the time it was 2005, the resistance increased up to 71% leading to an increase in the resistance

over 90% by the end of 2009 [7]. So it can be said that as the population grows, the demand and supply rate for rapid and feasible treatment increase. Antibiotics are more like the daily necessity of food for many South Asian under developed countries, even in the worst case of mild viral fever or mild flu, people tend to rely on an antibiotic and thus pharmaceutical companies have hit a jackpot.

The above study has been evident about one thing that even though clinical strain of *S.typhi* show full resistance against Oxacillin, Erythromycin, Nalidixic acid and Cefoxitin, the natural strains have showed full resistance against Oxacillin only. It can be said that one of the reasons why Oxacillin seemed to be ineffective against both clinical and natural isolates is that maybe it was never used as a medication against enteric infections in Bangladesh. The study was successful in a sense that desired results were obtained, and as expected clinical strains showed significant resistance when compared with the natural isolates, rightfully because natural isolates are still exhibiting in the environment and have not undergone the treatment procedures with several courses of antibiotics. Moreover, one noticeable outcome of this entire study was that the natural isolates had given some unknown bands (denoted by black arrows) of proteins after the SDS gel run which was seen to be absent in case of the proteins bands obtained for clinical isolates. It can be elaborated that most probably due to spontaneous mutation or being under antibiotic experiments for a long time the clinical isolates have acquired a different genetic makeup which resulted in the alteration of their protein profile, the proteins which could be the possible target for destruction by drugs or could be channels for the transport of the drugs to the target sites. However, it can be assumed that those proteins are still intact in the natural strains and hence they showed significant sensitivity against the antibiotics.

The method used for the study has been a popular one and has been successful in leading many research studies related to protein separation and purification. The method was chosen in order to specify the difference in the protein profiles between the clinical and natural isolates, and it was mildly accomplished to with the derivation of some unknown protein bands and then interpolating the molecular weights of these proteins from the graph plotted with the molecular weights of the known proteins. Usually proteins can be surface proteins, and present as lipoproteins or glycoproteins which leads to huge molecular weights and such factors can lead to significant errors and false results.

In order to minimize the consequences of antibiotic resistance and to find out the possible causes and effects of such developing patterns of resistance, it is essential to monitor the resistance pattern in our local society. This will not only validate the discarding of those antibiotics which are already ineffective, but will also increase the probability of supplying newer and more effective antibiotics. It will be vital to lessen the emergence of such drug resistant pathogens and also their spread amongst the population. This has been the sole purpose and heart behind this study and to some extent it was accomplished with at least an idea and conclusion about how the drug resistant pathogens are spreading and which common antibiotics are no more lethal to finish off the infections. Controls must be taken to halt the over usage of the drugs which are still effective, such as creating awareness by publishing as many research articles as possible and also by conducting such research studies variable times on different organisms and in different areas to jot down the overall condition and consequences of such drug resistant infections in whole Bangladesh.

Chapter 5: Conclusion

In order to achieve a proper and meticulous treatment of the infectious diseases and a more or less secured healthy environment in the country, a good, informative and representative data on the multi-drug resistance and development of newer antibiotics in the country is essential. Programmes on surveillance, databases on the mortality and morbidity rates should be taken as crucial steps in order to slow down the emergence of such drug resistant organisms. Regulation of the use of antibiotics in the hospitals and communities should be slowed down. Urgent steps can be taken to determine the total period of compliance therapy and reasons of non-compliance in low-income and rural populations. Also the prevalence of antimicrobials usage can be determined so that more effective ways are found out to contain the development of antibiotic resistance. Since we are immune to a life with antibiotics and is dependent on such medications for a more healthy and perfect life, it resides within our conscience and our desire to take steps or at least try and educate our newer generation through social media, or through text book studies to secure the futuristic health of our country, develop more attraction towards natural process of disease remedy. Modernization will lead to newer discoveries and newer drugs too, but the root cause of such issues of drug resistance will still sustain unless the producers find a way to warn their consumers about their over usage or unless the consumers themselves do not get alert about their future health and be more habituated in consuming medications only when prescribed by the doctor.

References

1) Antibiotic resistance—the need for global solutions, LIDC. 2013.
2) Cappuccino, J.G. and Sherman N. 1996. Microbiology - A Laboratory Manual, 4th Ed. The Benjamin/ Cummings Publishing Co., Inc., Menlo Park, California. pp. 13-182.
3) Faiz MA, Rahman MR. Rational antimicrobial use. Journal of Chittagong Medical College Teacher Association. 2004; 15(1&2): 1-3.
4) Ferrecio C. Benigh bacteremia caused by *Salmonella typhi* and *paratyph*i in children younger than 2 years. Journal of Paediatrics 1984; 104 : 899-901.
5) Hema, T. A., Arya. A.S, Subha. S, John. R. K and Divya. P.V. 2013. Antimicrobial activity of five south Indian medicinal plants against clinical pathogens. Int .J. Pharm. Bio. Sci. Vol. 4(1). pp. 70 – 80
6) Hossain MA, Rahman M, Ahmed QS, Malek MA, Sack RB, Albert MJ, Increasing frequency of methicillinam-resistant shigella isolates in urban Dhaka and rural Matlab, Bangladesh: a 6 year observation. Journal of Antimicrobial Chemotherapy. 1998; 42: 99 – 102.
7) Islam KMS. ESBL-a therapeutic challenge. Bangladesh Journal of Medical Microbiology. 2009; 03(01): 1 – 3.
8) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition, CLSI. 2012. Vol. 32 No. 2.
9) Nathan, C., and Cars, O. 2014. Antibiotic Resistance - Problems, Progress, and Prospects. The New England Journal of Medicine. Vol. 371. pp. 1761-1763.
10) . Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. 2014.
11) William W. Hay, Jr. Anthony R. Haywood, Myron J. Levin, Judith M. Sondheimur, Current Pediatric Diagnosis and Treatment, 15th ed. Lange Medical Book/ McGraw-Hill 2001: 541-542.
12) World Health Organization. Overcoming antimicrobial resistance. Geneva: WHO, 2000.

Appendices

APPENDIX-I

Media composition

The composition of the media used in the present study has been given below. Unless otherwise mentioned, all the media were autoclaved at 121^{0}C for 15 min.

1. **Nutrient Agar (Himedia,India)**

Ingredients	Amounts (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	15.0

2. **Nutrient Broth (Oxoid, England)**

Ingredients	Amount (g/L)
Lab-lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0

3. T1N1 soft agar

Ingredients	Amount (g/L)
Tryptone	0.6 g
Sodium chloride	0.3g
Agar	0.42 g

4. Simmon's citrate agar (Oxoid, England)

Ingredients	Amount (g/L)
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	0.2
Ammonium phosphate	0.8
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bacto brom thymol blue	0.08

5. MR-VP broth

Ingredients	Amount (g/L)
Peptone	7 g
Dextrose	5 g
Potassium phosphate	5 g

6. Triple sugar iron agar (Himedia, India)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	10.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous sulfate	0.20
Sodium thiosulfate	0.30
Casein enzymatic hydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0

7. Xylose Lysine Deoxycholate agar (Himedia, India)

Ingredients	Amount (g/L)
L- lysine	5.0
Lactose	7.50

Sucrose	7.50
Xylose	3.50
Sodium chloride	5.0
Sodium deoxycholate	2.50

8. Phenol red (Lactose, Dextrose, Sucrose) Broth

Ingredients	Amount (g/L)
Trypticase	0.4
Lactose	0.2
Sucrose	0.2
Dextrose	0.2
Sodium chloride	0.2
Phenol red	0.00072
Final pH	7.3

9. Mueller-Hinton agar

Ingredients	Amount (g/L)
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

APPENDIX-II

Buffers and reagents

1. Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4 and 2.0 g of KH2PO4 in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 liter by distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

2. Kovac's reagent

5 g of para-dimethylaminobenzaldehyde was dissolved in 75 ml of amyl alcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4oC.

3. Methyl red reagent

0.1 g of methyl red was dissolved in 300 ml of 95% ethyl alcohol. Then distilled water was added to make the final volume 500 ml. This reagent was covered with aluminum foil and stored at 4°C.

4. Barritt's reagent

Solution A

5 g of alpha-naphthol was dissolved in 95% ethanol. This solution was covered with aluminum foil and stored at 4°C.

Solution B

40 g of KOH was dissolved in distilled water. The solution became warm. After cooling to room temperature, creatine was dissolved by stirring. Distilled water was added. This solution was covered with aluminum foil and stored at

5. Oxidase reagent

100 mg of N,N,N1,N1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4°C.

APPENDIX-III

SDS-PAGE Reagents

1) 30% Acrylamide : (for 100ml)

Acrylamide	29.0 gm
Bis- Acrylamide	1.0gm
DH_2O	100ml

2) 1.5 M Tris (pH 8.8) : (for 100ml)

1.5 M Trisma base	18.17 gm
SDS	0.4 gm
DH_2O	100 ml

0.5 M Tris (pH 6.8) : (for 100ml)

1.5 M Trisma base	6.1 gm
SDS	0.4 gm
DH_2O	100ml

3) 10% SDS Solution: (for 100ml)

SDS	10 gm
DH_2O	100 ml

4) 10% APS (Ammonium persulphate) : (for 10ml)

APS	1 gm
DH_2O	10 ml

5) 1x TGS Running Buffer: (for 2.5 L) pH 8.3

Trisma base	7.5 gm
Glycine	36 gm
10% SDS	25 ml
DH_2O	upto 2.5L

6) Staining: (for 100 ml)

Acetic Acid	10 ml
Methanol	10 ml
DH_2O	80 ml
Comassie Blue	0.1-0.2 gm

7) Destaining: (for 100 ml)

Acetic Acid	10 ml
Methanol	10 ml
DH_2O	80 ml

APPENDIX-IV

Instruments

The important equipments used through the study are listed below:

❖ Autoclave	SAARC
❖ Freeze (-20°C)	Siemens
❖ Incubator	SAARC
❖ Micropipette (10-100µl)	Eppendorf, Germany
❖ Micropipette (20-200µl)	Eppendorf, Germany
❖ Oven, Model:MH6548SR	LG, China
❖ pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
❖ Refrigerator (4°C), Model: 0636	Samsung
❖ Safety cabinet Class II Microbiological	SAARC
❖ Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
❖ Vortex Mixture	VWR International
❖ Water bath	Korea
❖ Weighing balance	ADAM EQUIPMENT™, United Kingdom